THE ROLE OF *SALMONELLA* ENTERITIDIS PATHOGENICITY ISLAND-1 IN THE COLONIZATION OF CHICKENS

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ABSTRACT

Salmonella enterica serovar Enteritidis (*S*. Enteritidis) is a major cause of gastrointestinal disease in humans worldwide that is mainly associated with the consumption of contaminated poultry meat and eggs. During the course of infection, *S*. Enteritidis uses two Type 3 Secretion Systems (T3SS), one of which is encoded by *Salmonella* Pathogenicity Island-1 (SPI-1). SPI-1 plays a major role in the invasion process.

In order to study the role of SPI-1 in the colonization of chickens, we constructed deletion mutants affecting either the complete SPI-1 region (40 kb) or *invG*, a single gene located on this pathogenicity island. The mutants were impaired in the secretion of effector proteins and were less invasive compared to the wild type strain in polarized Caco-2 cells. Similarly, when chicken cecal and small intestinal explants were co-infected with the wild type and ΔSPI-1 mutant strains we found that the ΔSPI-1 mutant strain was less invasive relative to the wild type strain. Oral challenge of 1-week-old chickens with the wild type or ΔSPI-1 strains demonstrated that there was no difference in chicken cecal colonization. However, systemic infection, measured as levels of *Salmonella* in the liver and spleen, was delayed in birds that were challenged with the ΔSPI-1 strain. This demonstrates that SPI-1 facilitates systemic infection but is not essential for invasion and systemic spread of *S*. Enteritidis in chickens.

Based on the above results, we examined the effect of sera against SPI-1 T3SS components to *S*. Enteritidis invasion. Anti-SipD serum protected Caco-2 cells against entry of wild type *S*. Enteritidis, but not against invasion of a mutant strain lacking *sipD*. On the other hand, sera against InvG, PrgI, SipA, SipC, SopB, SopE and SopE2 did not affect *S*. Enteritidis entry. To illustrate the specificity of anti-SipD mediated inhibition, SipD specific antibodies were depleted from the serum. Depleted serum restored the invasion of *S*. Enteritidis, demonstrating that the SipD protein may be an important target in blocking SPI-1 mediated virulence.

To determine if SPI-1 T3SS proteins were protective against *S*. Enteritidis oral challenge, chickens were vaccinated subcutaneously twice at 14 and 28 days of age with PrgI and SipD. The results indicate that these proteins induce strong IgG antibody responses and confer significant protection against infection of the livers in vaccinated birds. In another study, we vaccinated hens with selected SPI-1 T3SS proteins to determine if their progeny could be protected from *S*. Enteritidis oral challenge. The proteins induced strong antibody responses but did not affect the levels of the challenge strain in the ceca or internal organs of the vaccinates. Taken together, our results establish that *S*. Enteritidis SPI-1 is an important virulence factor in chickens and that the proteins associated with this T3SS may form components of a subunit vaccine used for protection against colonization by *S*. Enteritidis in poultry.

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DEDICATION

This thesis is dedicated to:

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LIST OF ABBREVIATIONS

AP-1	Activator Protein 1
BBS	Borate Buffered Saline
BGA	Brilliant Green Agar
Caspase	Cysteine dependent aspartate specific protease
CFU	Colony forming unit
ddH2O	Double-distilled water
DMEM	Dulbecco's Modified Eagles Medium
ELISA	Enzyme Linked Immunosorbent Assay
E. coli	*Escherichia coli*
ERK	Extracellular regulated kinase
FBS	Fetal Bovine Serum
Fis	Inversion stimulation
Fur	Ferric uptake regulator
GAP	GTPase Activating Protein
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GTP	Guanosine Triphosphate
HACCP	Hazard Analysis Critical Control Point
IFNγ	Interferon-gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHF	Integration Host Factor
IL	Interleukin
LB	Luria Bertani
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
Mbp	Mega base pairs
MOI	Multiplicity of Infection
NAP	Nucleoid Associated Proteins

NF-κB	Nuclear Factor Kappa B
PAMP	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
PEF	Plasmid Encoded Fimbriae
PMN	Polymorphonuclear Leukocytes
SCV	*Salmonella* Containing Vacuole
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electophoresis
S. Enteritidis	*Salmonella enterica* serovar Enteritidis
SPF	Specific Pathogen Free
SPI	*Salmonella* Pathogenicity Island
S. Typhimurium	*Salmonela enterica* serovar Typhimurium
TCA	Trichloro-acetic acid
TER	Trans-epithelial resistance
TGF	Transforming Growth Factor
TLR	Toll-Like Receptor
T3SS	Type 3 Secretion System
VIDO	Vaccine and Infectious Disease Organization
WASP	Wiskott-Aldrich Syndrome Protein
WT	Wild Type *S*. Enteritidis

1.0 LITERATURE REVIEW

1.1 Salmonella

1.1.1 Nomenclature

The bacterial genus *Salmonella* was named following the discovery of this pathogen by the United States Department of Agriculture (USDA) veterinary bacteriologist Dr. Daniel E. Salmon (1850-1914) [1]. *Salmonella* is a Gram negative, facultative intracellular, rod-shaped (0.7-1.5 μm wide x 2-5 μm long) and non-spore forming member of the enterobacteriacae family. *Salmonella* species are mostly peritrichously flagellated and motile. The optimal temperature for growth of this pathogen is 37° Celsius [2]. The genus *Salmonella* is divided into two species, *Salmonella bongori* and *Salmonella enterica*. *S. enterica* consists of six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *indica*, and *houtenae* [3]. *S. enterica* subspecies *enterica* is mainly associated with infection in warm blooded animals, while the other subspecies and *Salmonella bongori* rarely cause infection in these animals [4, 5]. *S. enterica* subspecies *enterica* consists of more than 2000 serovars [6] which are classified based on the O factor (O antigen) of the lipopolysaccharide (LPS) and the H antigens of the flagella [7].

1.1.2 *Salmonella* Enteritidis

S. enterica subspecies *enterica* serovar Enteritidis (*S*. Enteritidis) has a genome size of approximately 4.69 Mbp [8] and is one of the most prevalent serovars with respect to human *Salmonella* infections world wide [9]. The consumption of contaminated meat (mainly poultry) and eggs are major sources of *S*. Enteritidis human infections [10]. Typically human infections result in a localized gastrointestinal disease which is characterized by a massive influx of neutrophils (hallmark of these infections) in the terminal ileum and colon (enterocolitis) resulting in nausea, vomiting and diarrohea [11]. Mostly, such infections are self-limiting and clear within 3-5 days. However, *S.* Enteritidis infections can also cause systemic disease, requiring antibiotic treatment or even hospitalization. On rare occasions, *S.* Enteritidis infections can also lead to the

development of pain in the joints, eye irritation and painful urination (referred to as Reiter's Syndrome) [12]. It is estimated that there are approximately 1.4 million cases of human salmonellosis in the United States annually [12], while in Canada the number of cases has been estimated to be close to 0.5 million every year [13]. The impact of these infections is massive on the U.S. and Canadian economies due to medical care, loss of work and death [14].

1.1.3 Virulence factors

1.1.3.1 Flagella

Salmonella species express flagella on their surface, which are organelles whose primary role is cell propulsion [15]. Rotation of the flagellar filament propels the bacterial cell either in a forward motion or random reorientation, depending on the chemical stimulus in the environment (chemotaxis). Although motility is its primary function, the flagella have also been shown to secrete SPI-1 T3SS effector proteins, SptP and SopE, under certain conditions [16].

The major components of the flagellum that are exposed on the surface consist of the proteins FliD (cap), FliC (filament – about 5 to 10 μm in length) and FlgE (hook – about 55 nm in length). The basal structure of the flagellum consists of an outer membrane anchored L ring (FlgH), a periplasmic rod, a periplasmic P ring (FlgI), an inner membrane MS ring (FliF), Mot proteins that constitute the stator element of the motor (Mot A and MotB), C ring (FliM and FliN) and a type 3 flagellar protein export apparatus (FlhA, FlhB, FliO, FliP, FliQ, FliR) [17]. The motility of the bacterial flagellum is powered by the proton motive force [16], while the assembly of the flagellar apparatus is powered by an ATPase known as FliI. The components of the basal structure of the flagellum share sequence homology to inner membrane components of T3SS, while components of the surface structure share little or no homology to T3SS components [17].

Salmonella flagella have been shown to be important in adherence to and invasion of epithelial cells in vitro. Using mouse models of infection, it has been shown that flagellar mutants are not impaired in virulence, while in the bovine model of infection, the aforementioned mutants were found to cause less inflammation [18]. Hence, this suggests that flagella are not required, but are important for virulence in the mouse and bovine models of infection. However, recently it has been suggested that flagellin (major monomeric subunit of the filament) is a powerful inducer of host innate immunity via the toll-like receptor 5 (TLR5) [19].

1.1.3.2 Fimbriae

Salmonella species contain hair-like structures present on their surfaces which are composed of polymeric protein subunits known as fimbriae. These structures play an important role in adhesion of the bacterium to the intestinal epithelium and hence form important virulence factors [20]. To date, 13 fimbrial loci have been identified [6], four of which are well defined in *S*. Enteritidis. These operons correspond to Type I fimbriae, SEF 14, thin aggregative fimbriae and plasmid encoded fimbriae [21]. The genes corresponding to type I fimbriae are located at centisome 15 and are encoded by the *fimAICDHF* operon. FimA forms the major structural monomer and these fimbriae are about 7-8 nm in diameter and up to 100 nm long. They are peritrichously distributed and bind α-D-mannose receptors on eukaryotic cells. SEF14 fimbriae are encoded by the *sef* operon and are less than 3 nm in diameter. SefA forms the major structural component and plays an important role in serological tests used for the detection of *S*. Enteritidis. Thin aggregative fimbriae (also knows as SEF 17 or curli) are located between centisome 40 and 43.3 of the *S.* Enteritidis chromosome and are encoded by the *agfBAC* operon. The fimbriae are approximately 3-4 nm wide and AgfA forms the major structural unit. A fourth type of fimbriae is the plasmid-encoded fimbriae (PEF) whose genes are encoded by the *pefBACD* operon found on a *Salmonella* virulence plasmid (described below). PEF are thought to play an important role in attachment to intestinal epithelial cells in vivo. However, the tissue specificity of type I fimbriae, SEF 14 and SEF 17 has not been determined [20, 21].

1.1.3.3 Plasmids

S. Enteritidis strains carry a 59 kb virulence plasmid which is also present in other serovars (although the sizes may differ). The main virulence associated genes include *spvRABCD* (Salmonella plasmid virulence) genes, *rck* (resistance to complement killing), *pef* (plasmid-encoded fimbriae), *srgA* (SdiA-regulated gene, putative disulphide bond oxireductase) and *mig-5* (macrophage-inducible gene encoding for putative carbonic anhydrase). The *spv* genes play an important role in macrophage survival and hence are important in causing systemic disease. The *rck* gene products increase the resistance of *S.* Enteritidis to complement mediated killing while the *pef* genes encode for a class of fimbriae described above. The roles of the *srgA* and *mig-5* genes in virulence are not very well known [22]. Certain *Salmonella* species also carry other plasmids which confer antibiotic resistance [22] and the presence of such plasmids makes these strains difficult to treat in human infections.

1.1.3.4 Type 3 Secretion Systems and Pathogenicity Islands

In order to inject virulence factors directly into host cells, *S*. Enteritidis uses a secretion pathway that makes use of a complex syringe-like system known as the Type 3 Secretion System (T3SS), which is also used by other gram negative pathogens. The T3SS is composed of more than 20 proteins that form a structure which spans the inner membrane, periplasmic space including peptidoglycan layer, outer membrane, and extracellular space. Bacterial virulence factors (known as effector proteins) are secreted and translocated into eukaryotic cells via a single step in a contact dependent manner, which results in the manipulation of host cellular architecture [23].

S. Enteritidis uses two T3SSs during the process of infection. These molecular syringes are encoded by regions of the chromosome known as *Salmonella* Pathogenicity Islands (SPI) which are thought to have been acquired during evolution via horizontal gene transfer. One of the T3SSs is encoded on Salmonella Pathogenicity Island 1 (SPI-1), while the other T3SS is located on SPI-2. The SPI-1 T3SS plays a very important role in invasion and inflammation during the process of infection. On the other hand, the SPI-2 T3SS is crucial for intracellular survival and systemic spread of the bacterium [24-26].

To date, twelve SPI's have been discovered of which SPI-1 to SPI-5 are well characterized [27]. SPI-3, SPI-4 and SPI-5 are mainly involved in growth and survival of the bacteria in the host species. The SPI's are characterized by a lower GC content (between 37 and 47 %) relative to the rest of the bacterial chromosome (approximately 52 %), are flanked by direct repeats or insertion sequences (IS) and are generally conserved between the different *Salmonella* serovars [26].

1.2 *Salmonella* Pathogenicity Island-1 Type 3 Secretion System

The SPI-1 T3SS is located at centisome 63 of the *Salmonella* chromosome and is about 40 kb in size (Figure 1.1) [25]. This T3SS is important for invasion of intestinal epithelial cells. During the process of infection, *S*. Enteritidis injects several effector proteins into the host cell which results in actin polymerization and membrane ruffling. This ultimately leads to uptake of the bacterium. Hence *S.* Enteritidis is able to breach the intestinal surface to gain access to underlying tissue and spread systemically [24, 26, 28].

1.2.1 Structure

The SPI-1 T3SS apparatus (Figure 1.2) belongs to an injectisome family which includes T3SS structures from *Shigella flexneri*, *Bordetella pseudomallei* and *Yersinia enterocolitica* [17]. The surface structure of this apparatus consists of a needle like structure and a translocon (involved in pore formation). The SPI-1 needle is formed by PrgI, while the translocon consists of SipB, SipC, SipD. The basal structure of this apparatus consists of outer membrane and inner membrane proteins that are connected by a periplasmic protein. InvG is the outer membrane protein and forms a pair of rings [29]. This protein is a member of the secretin family of transporters which are also found in other secretion systems [17, 29, 30]. The InvG secretin is connected by PrgJ, a periplasmic protein, to the inner membrane structure that mainly consists of PrgH and PrgK [31]. InvA, SpaP, SpaQ, SpaR and SpaS form part of the export machinery that is connected to PrgH and PrgK [32]. The SPI-1 apparatus also contains a motor-like energy source known as the "ATPase" (InvC) that provides the energy required for the secretion of proteins through this channel [33].

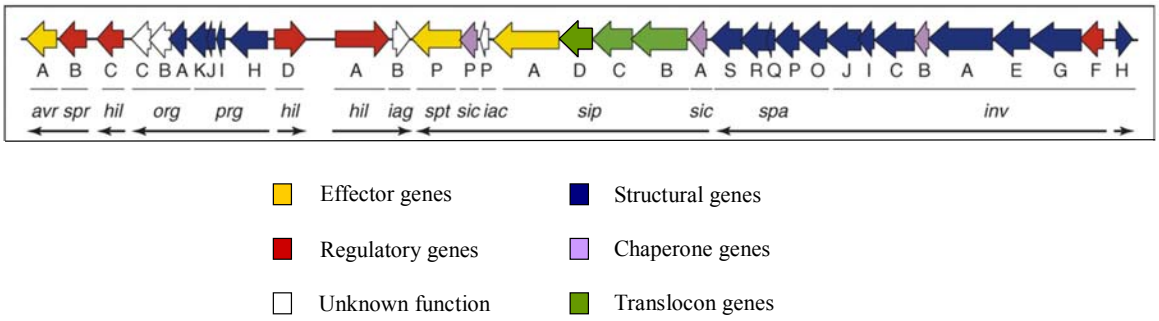


Figure 1.1: Schematic representation of Salmonella Pathogenicity Island-1 genes. Arrows below the gene names represent transcripts. The *sitABCD* operon downstream of *avrA* is not shown since it does not play a part in invasion. Diagram was adapted and modified from [34].

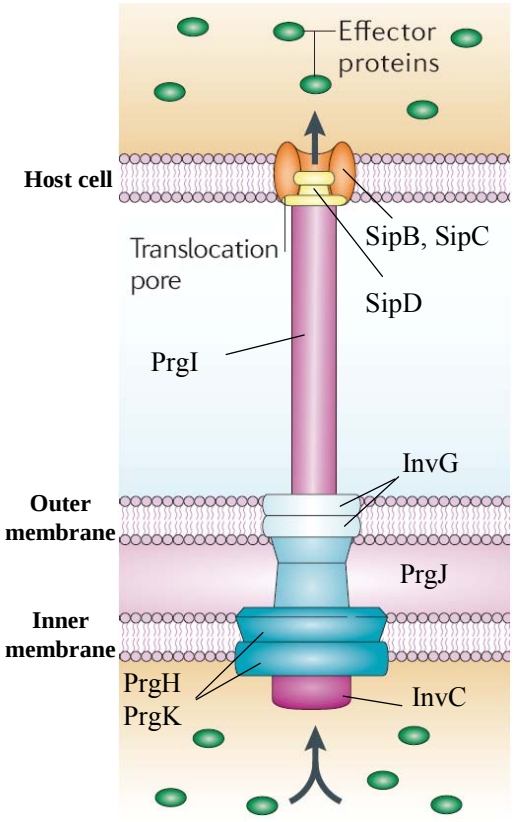


Figure 1.2. Structure of the *S*. Enteritidis SPI-1 Type 3 Secretion System. Diagram was adapted and modified from [17].

1.2.2 Assembly

The first step in the assembly process is the export of the inner membrane proteins PrgH and PrgK, and the outer membrane secretin InvG (which requires the help of a secretin specific protein InvH), by the general secretory (sec) pathway. This step is thought to occur in a sec-dependent fashion since all the aforementioned proteins contain N-terminal sec-dependent signal sequences [29, 35]. Moreover, proteins exported through this pathway are transported into the periplasm by two heterotrimeric complexes and an accessory protein found in the inner membrane (SecYEG, SecDFYajC and SecA, respectively) [36]. Once the assembly of PrgH, PrgK and InvG is completed, the export apparatus (InvA, SpaP, SpaQ, SpaR and SpaS) and the ATPase (InvC and SpaL), which conists of inner membrane components is formed. This completes the formation of the basal structure and is followed by the secretion of PrgI (needle) and PrgJ (inner rod) through the newly formed export apparatus in a type 3 dependent manner [29]. As the needle reaches a certain length (approximately 80 nm) another protein, InvJ, stops the export of PrgI [29, 37]. The SipD protein is secreted through this system, forms the tip of the PrgI needle and plays an important role in mediating *Salmonella* species attachment to host cells in order to secrete effector proteins. SipD is present at the tip of the needle complex prior to contact with host cells, while the other members of the translocase (SipB and SipC) are exposed after contact with host cells [38].

1.2.3 Secretion

Salmonella species secrete many virulence factors (known as effector proteins) through the SPI-1 T3SS that are translocated into host cells. Contact with host cells [39] and the presence of cholesterol (host cell membrane) [40] are the main triggers of effector protein secretion. This is based on evidence from studies involving *Yersinia pseudotuberculosis* and *Shigella flexneri*, respectively. In addition, it has recently been proposed that the needle acts as a sensor and causes a change in the structure of the needle once in contact with host cells, leading to the secretion of effector proteins [41]. To date, at least 15 effectors (table 1.1) have been identified that are secreted using the SPI-1 T3SS.

Table 1.1: List of effector proteins secreted by the *S*. Enteritidis SPI-1 T3SS. Table was adapted and modified from [28].

Effector protein	Gene location	Function	Reference
AvrA	SPI-1	Inhibits inflammation and apoptosis, stabilizes epithelial cell tight junctions.	[42-44]
SipA	SPI-1	Promotes actin polymerization by binding to actin filaments, induces infiltration of PMN across the intestinal epithelial layer, maintains perinuclear position of SCV, stimulates intracellular replication.	[45-49]
SipB	SPI-1	Forms part of translocon, binds to cholesterol and is involved in triggering secretion of effector proteins, contributes to inflammation and macrophage cell death.	[50-53]
SipC	SPI-1	Forms part of translocon, enhances actin polymerization	[54]
SipD	SPI-1	Forms part of translocon structure and the tip of the secretion system, is involved in mediating attachment to epithelial cells.	[28, 38]
SopA	outside SPI-1	Induces migration of PMN's across the intestinal epithelial layer, allows *Salmonella* to escape from the SCV.	[55, 56]
SopB	SPI-5	Promotes actin polymerization, enhances membrane fission, induces expression of pro-inflammatory cytokines like IL-8, plays a role in SCV maturation.	[28, 57-62]
SopD	outside SPI-1	Enhances membrane fission	[59]
SopE	Bacteriophage	Promotes actin polymerization through GEF activity, induces inflammation, enhances SCV maturation.	[28, 60, 61, 63]
SopE2	Bacteriophage	Promotes actin polymerization through GEF activity, induces inflammation.	[60, 61, 63]
SptP	SPI-1	Allows cell to regain its original architecture following invasion, down regulates inflammatory response.	[64-67]
SlrP	Outside SPI-1	Causes cell death *in vitro*.	[68]
SspH1	Bacteriophage Gifsy-3	Inhibits NF-κB expression resulting in reduced inflammation.	[69, 70]
SteA	outside SPI-1	Localizes to the Golgi network in infected cells.	[71]
SteB	outside SPI-1	Unknown.	[71]

After the secretion of effector proteins into host cells by *Salmonella*, the host cellular architecture is modulated; leading to the formation of membrane ruffles and lamellipodial extensions, which triggers bacterial internalization. *Salmonella* species employ the concerted action of 7 effector proteins to directly (SipA and SipC) or indirectly (SipB, SopB, SopD, SopE and SopE2) modulate the dynamics of actin filaments [28], which are the most dominant structural components in the lamellipodium [72].

The assembly of actin filaments is not a favourable process due to the instability of actin dimers and trimers [72]. Therefore, SipA promotes actin polymerization by binding to actin filaments and reducing the critical concentration for actin assembly as well as stabilizing the filaments [73]. In addition, SipA also enhances the activity of T-plastin (a host cell actin bundling protein) and prevents the binding of ADF/cofilin (actin depolymerising proteins) to actin filaments [45, 46]. SipC on the other hand, forms part of the SPI-1 translocon and enhances the formation of actin trimers and bundles actin filaments [54]. Another SPI-1 effector protein, SipB, forms part of the SPI-1 apparatus translocon and has not been implicated with actin polymerization. However, SipB binds to cholesterol in the plasma membrane and it is thought that this may also represent one of the steps in triggering the secretion of SPI-1 effector proteins [53].

Salmonella also causes the polymerization of actin filaments indirectly through other pathways. This involves the activation of Cdc42 and Rac-1, which are members of the Rho subfamily of actin-organizing small guanine binding proteins. These proteins are active when they are bound to guanosine triphosphate (GTP) and inactive when guanosine diphosphate (GDP) is bound to them. The activity of Cdc42 and Rac-1 are controlled by two regulatory proteins: guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs catalyze GTP loading to Cdc42 and Rac-1, while the latter (GAPs) enhance GTP hydrolysis to GDP [65, 74]. The SPI-1 effector protein SopE acts as a GEF through activation of Cdc42 and Rac1 by catalyzing the exchange of GDP with GTP, while SopE2 performs a similar function mainly in

association with Cdc42 [63]. *Salmonella* also activates Cdc42 and Rac-1 indirectly through the activity of SopB (phosphatidyl inositol phosphatase activity). SopB hydrolyzes many phosphatidyl inositol phosphates and inositol phosphates, generating secondary messengers (such as phosphatidyl inositol-4,5-diphosphate), which in turn also activate Cdc42 and Rac-1 [57, 58]. Hence, Cdc42 and Rac-1, in their active forms, activate members of the Wiskott-Aldrich Syndrome protein (WASP) family. Proteins of the WASP family recruit and activate the actin-related protein-2/3 (Arp2/3) complex, which plays a very important role in actin nucleation, branching and cross-linking actin filaments [61, 72, 75]. Another SPI-1 effector protein, SopD, enhances membrane fission and macropinosome formation, along with SopB, at the site of bacterial invasion. However, the exact mechanisms by which SopD performs its role are not known [59].

Following bacterial invasion, *Salmonella* employs SptP (SPI-1 effector protein) which has GAP activity. SptP hydrolyzes GTP to GDP on Cdc42 and Rac1, thus deactivating these proteins. Hence, this counteracts the effects of SopB, SopE and SopE2 allowing the host cell to regain its original architecture [64, 65].

The secretion of effector proteins by the SPI-1 T3SS also results in the modulation of host pathways, leading to inflammation. SopB (as well as SopE and SopE2) mediated activation of Cdc42 results in the stimulation of different mitogen activated protein kinase (MAPK) cascades (Erk, Jnk and p38 pathways) that activate transcription factors: activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB). This induces the expression of pro-inflammatory cytokines, like IL-8, causing an influx of polymorphonuclear leukocytes (PMN), and eventually causing diarrhoea [60, 61]. Similarly, the SPI-1 effector protein SipA, induces signalling pathways that activate protein kinase Cα, resulting in the secretion of PMN chemoattractant hepoxillin A3. This enhances the infiltration of PMN across the intestinal epithelial layer which contributes to intestinal inflammation and diarrhoea [47, 48]. The influx of neutrophils in the intestine is accompanied with fluid secretion by intestinal epithelial cells, which is regulated by chloride secretion. Increased levels of intracellular inositol phosphate ($Ins[1,4,5,6]P_4$), as a result of SopB activity, affect different cellular pathways leading to an influx of

chloride ions. Hence, this process also contributes to the diarroheal response in the host [47]. Another SPI-1 effector protein, SipB, also contributes to the inflammatory response by inducing rapid macrophage cell death. This occurs when SipB binds to and activates a cysteine-dependent aspartate-specific protease (caspase) known as caspase-1 in macrophages [50]. Activated caspase-1 causes the activation and release of pro-inflammatory cytokines IL-1β and IL-18, which stimulate the recruitment of immune cells and enhance the inflammatory response. Further, activated caspase-1, through other downstream processes, causes the formation of membrane pores leading to the release of cellular contents and cell lysis [51, 52]. The recruitment of PMNs across the intestinal epithelium is also promoted by the SPI-1 effector protein SopA, through its E3 ubiquitin ligase activity. Ubiquitination is the process of attaching ubiquitin (regulatory protein) to proteins, so that the proteins are either labeled for degradation or directed to different cellular compartments. It controls a variety of cellular processes and involves a cascade of enzymes including E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin protein ligase). SopA mimics host E3 enzymes by binding host E2 enzymes and catalyzing the transfer of ubiquitin to itself. Thereafter, ubiquitinated SopA induces migration of PMNs across the intestinal epithelial layer [55, 56, 76].

The aforementioned inflammatory responses are suppressed by the SPI-1 effector proteins AvrA, SptP and SspH1. AvrA is a cysteine protease that possesses deubiquitinase and acetyltransferase activity. This effector removes ubiquitin (deubiquitinates) from IκBα and β–catenin (inhibitors of NF-κB signalling), preventing them from proteasomal degradation. Hence, IκBα and β–catenin are able to suppress NF-κB signalling, leading to reduced inflammation and apoptosis [42]. In addition, AvrA also acetylates certain residues of upstream activators of MAPKs (MAPK Kinase MKK4/7) preventing their subsequent phosphorylation. This leads to the inhibition of MAPK pathways resulting in reduced inflammation [43]. Moreover, AvrA has also been associated with stabilizing epithelial cell tight junction structure, which is disrupted during *Salmonella* invasion [44]. Similarly, the SPI-1 effector SptP employs its tyrosine phosphatase activity to dampen inflammatory responses. Tyrosine kinases play an

important role in the activation of MAPK pathways during *Salmonella* infection, which leads to the secretion of pro-inflammatory cytokines. SptP dephosphorylates proteins involved in the extracellular-regulated kinase (ERK) MAPK pathway, resulting in an inhibition of the pathways. Hence, this process lowers the inflammatory response [66, 67]. Likewise, SspH1, another SPI-1 effector protein, reverses the host inflammatory reaction to *Salmonella* infection through its E3 ubiquitin ligase activity by ubiquitinating protein kinase N1 (PKN1). Ubiquitinated PKN1 inhibits NF-κB dependent gene expression [69, 70].

SPI-1 effector proteins also play a role in processes following invasion (where SPI-2 effector proteins are the major players) including maturation and positioning of the *Salmonella* containing vacuole (SCV) and intracellular replication [6, 28]. SopB and SopE play important roles in maturation of the SCV, by recruiting cellular markers associated with early endosomes such as Rab5 (member of the Rab GTPase family). This in turn leads to the recruitment of other proteins (Vps34 and endosomal antigen-1) which results in the accumulation of phosphatidyl inositol 3 phosphate at the SCV membrane (important for SCV membrane maturation). SopB also prevents the SCV from entering the endosomal maturation pathway [28, 62]. As well, SopB mediated activation of a host kinase protein (Akt) prevents SCV phagosome-lysosomal fusion, thus allowing *Salmonella* to persist and replicate in the SCV [77]. The positioning of the SCV in close proximity to the nucleus (perinuclear position) is mediated in part by the SPI-1 effector, SipA, along with other SPI-2 effector proteins. SipA also plays a role in stimulating the replication of intracellular *Salmonella* [28, 49]. Another mechanism by which SPI-1 effector proteins are involved in late stages of infection deals with *Salmonella* escaping from the SCV. SopA has been implicated in allowing *Salmonella* to escape from the SCV through interaction with another host E3 ligase known as the RING finger protein with membrane anchor (HsRMA-1) [55].

In addition to the aforementioned SPI-1 effector proteins, recently other effector proteins have been discovered that are also secreted using this system. This includes, SlrP, SteA and SteB. However, these proteins are also secreted by the SPI-2 T3SS. SlrP

ubiquitinates mammalian thioredoxin (regulatory protein) which ultimately causes cell death in vitro [68]. Despite being secreted by both SPI-1 and SPI-2 systems, the expression of SlrP is induced by a SPI-1 regulator [78]. SteA and SteB are secreted by both the SPI-1 and SPI-2 T3SS. SteA localizes to the Golgi network in infected cells and is required for colonization of mouse spleens, while SteB does not appear to play a role in the colonization of mice. The regulation of SteA and SteB is not known [71].

In summary, SPI-1 effector proteins are involved in different functions including invasion, modulation of the inflammatory response and SCV maturation and positioning (Figure 1.3).

1.2.4 Regulation

The SPI-1 locus regulation is complex and involves local regulators, global regulators and environmental factors. The HilA protein (encoded on SPI-1) is one of the main local regulators of SPI-1 gene expression. This protein binds directly to promoters on the *inv-spa* and *prg-org* operons on SPI-1, which results in the activation of transcription. Read-through from the *inv-spa* operon also leads to the activation of InvF, a positive transcriptional regulator, and the *sic-sip* operon. InvF, along with SicA (chaperone), induces the expression of effector proteins located on SPI-1 (*sic-sip* operon) as well as non-SPI-1 effectors. Transcription of *hilA* is controlled by HilC, HilD and RtsA. Once HilD is expressed, it activates the transcription of *hilC* and *rtsA*. HilC and RtsA further enhance the expression of each other and themselves. Along with HilD, HilC and RtsA activate the transcription of *hilA*, which ultimately leads to the expression of SPI-1 genes (summarized in Figure 1.4) [34].

Many global regulators affect the expression of SPI-1 genes through the transcriptional activator HilD. One such system is the EnvZ/OmpR two component regulatory system. This system controls the expression of two major outer membrane proteins, OmpC and OmpF, in response to different stimuli including osmolarity. OmpC and OmpF form aqueous channels allowing passive diffusion of small molecules across the outer membrane. EnvZ acts as a sensor, monitoring conditions in the external

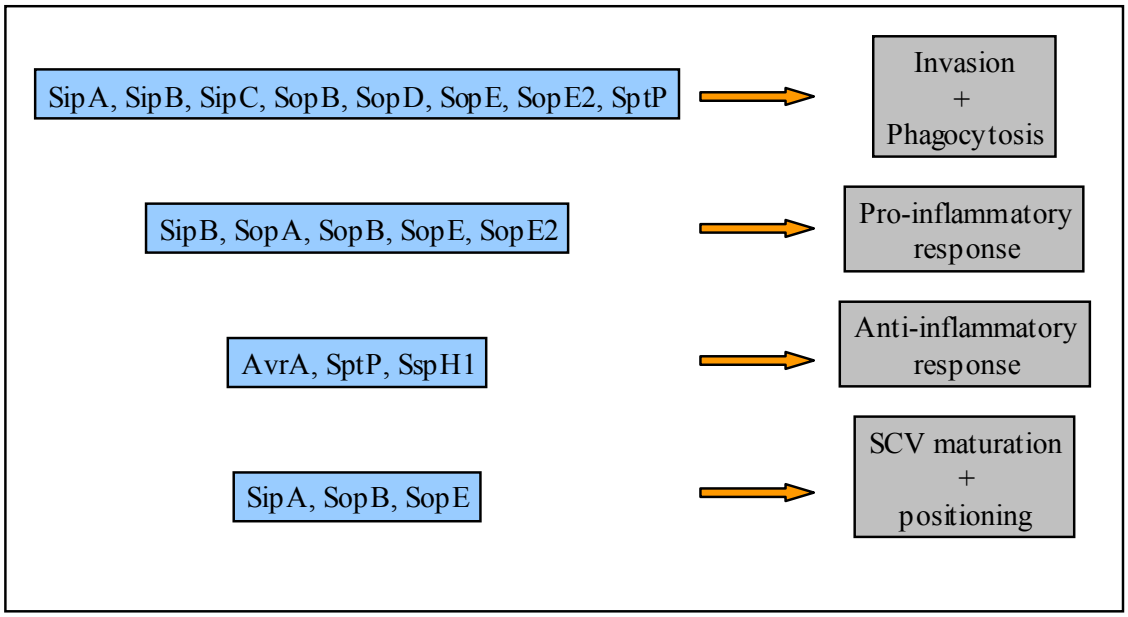


Figure 1.3. Summary of the main functions of *S*. Enteritidis SPI-1 T3SS effector proteins.

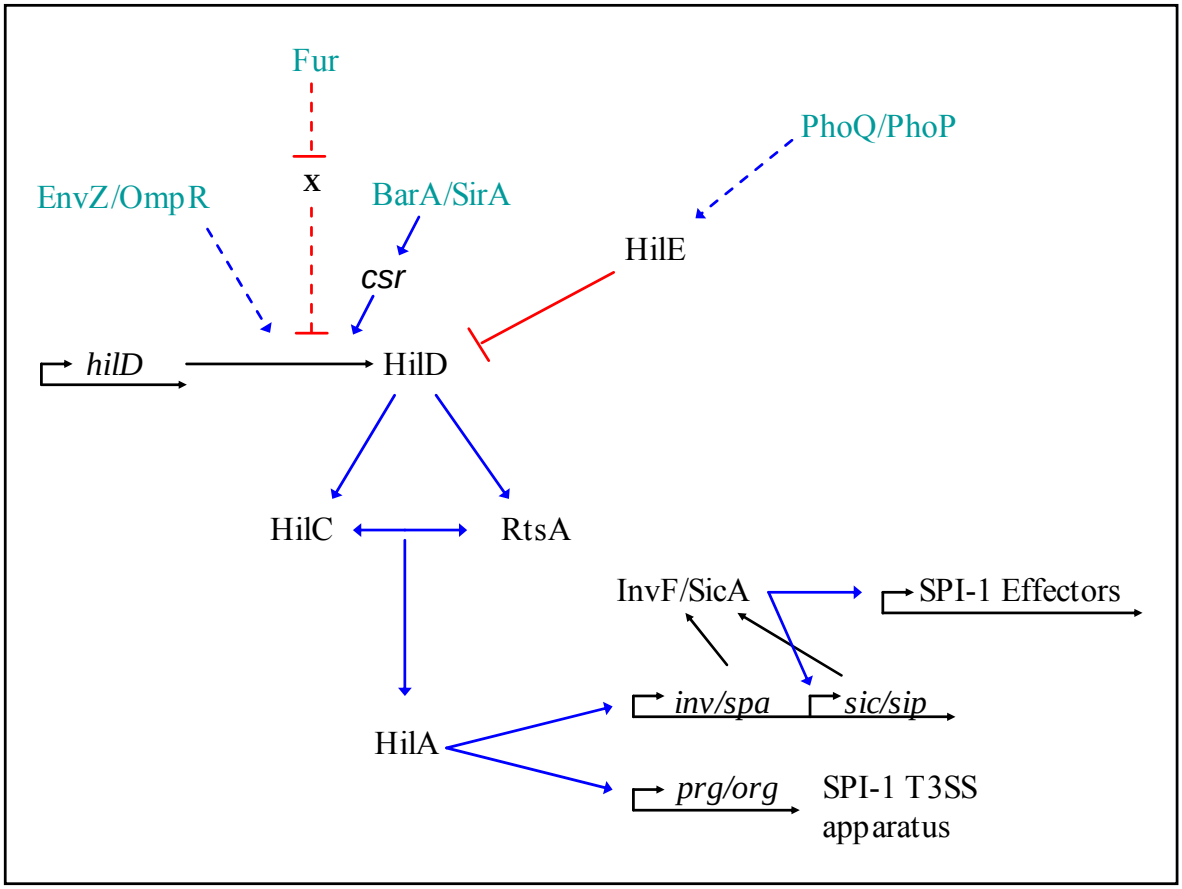


Figure 1.4. Regulation of the *S.* Enteritidis SPI-1 T3SS by local and global regulators. Blue arrows represent activation of gene expression. Red lines with blunt ends represent repression. Lines with dashes indicate that the exact mechanism of regulation is not known. Global regulators are in green while local regulators are in black. Diagram was adapted and modified from [34].

environment and transfers this information to OmpR through phosphorylation [79]. Once phosphorylated, OmpR induces the expression of *hilD* (along with the regulation of several other bacterial genes) which ultimately leads to the expression of SPI-1 genes [80]. A second global regulator of SPI-1 genes is the BarA/SirA two component regulatory system which regulates various genes involved in metabolism, motility, biofilm formation and invasion [34]. BarA is a sensor kinase which phosphorylates SirA in response to certain environmental signals (unknown so far). Phosphorylated SirA activates expression of two small RNA molecules *csrB* and *csrC*, which in turn prevent CsrA action (repressor of *hilD*). Hence, HilD expression is increased and this leads to increased SPI-1 gene expression [34, 81]. The ferric uptake regulator (Fur) is one of the most important iron dependent regulators in *Salmonella* and is another global regulator of SPI-1 gene expression. In response to high iron levels (as in the intestinal lumen), Fur (a repressor) binds to Fe^{2+} and represses the expression of several genes involved in the uptake of iron and other metals. In addition, Fur has shown to affect *hilA* activity (SPI-1 gene expression) by regulating HilD at the post-transcriptional level by either repressing a negative regulator of HilD or by controlling the level of HilD required to activate the *hilD* promoter and thus the expression of SPI-1 genes [82]. The PhoP/PhoQ two-component system is yet another global regulator of SPI-1 gene expression. PhoQ (sensor) activates PhoP in response to low levels of Mg^{2+} and Ca^{2+} (as in the intracellular and phagosomal environment) [25, 83]. This results in the activation of *hilE* expression, which represses HilD leading to the repression of SPI-1 gene expression [34]. This regulation is an important mechanism of switching off or lowering the expression of the SPI-1 T3SS genes, since they are not major players in intracellular survival and replication (summarized in Figure 1.4).

The expression of SPI-1 genes is also affected by several environmental signals. These environmental signals include pH, osmolarity, and the levels of oxygen and magnesium. Since the main site of invasion by *Salmonella* is the small intestine, the environmental conditions associated with it lead to increased expression of SPI-1 virulence genes. The pH level of the small intestine is near neutral and has been shown to increase expression of genes associated with the SPI-1 locus. Similarly, the osmolarity of

the small intestine is high which increases expression of SPI-1 genes, possibly through the OmpR/EnvZ two component system. The intestinal lumen is anaerobic, while the brush border is microaerophilic. Low oxygen conditions have been shown to elevate the expression of SPI-1 genes. Finally, high levels of magnesium in the extracellular environment of the small intestine and elsewhere in the host result in repression of the PhoP/PhoQ system which normally represses SPI-1 gene expression. Taken together, this demonstrates that SPI-1 regulation is fairly complex and involves several parameters that include local, global and environmental regulators [84, 85].

1.2.5 Role in Virulence

The role of the SPI-1 T3SS has been extensively studied in the bovine and murine (streptomycin-treated mice) models of infection [47]. These studies have been mainly performed with *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) which causes a similar non-typhoidal disease in humans and animal models. Oral infection of streptomycin treated mice and calves results in a massive neutrophil influx in the gastro-intestinal tract causing inflammation, which is analogous to the clinical and histological manifestations of the disease in humans. As well, in mice, the infection also spreads to internal organs causing systemic disease [11]. SPI-1 mutants have been shown to cause reduced inflammation and fluid influx in the aforementioned animal models, demonstrating that SPI-1 is critical for the intestinal form of the disease [47]. Moreover, recently it has been demonstrated that SPI-1 effector proteins are also synthesized during later stages of infection in mice, suggesting that SPI-1 may play a role in systemic disease [86]. In chickens, the function of SPI-1 is not as well characterized and has mostly been observed using *S.* Typhimurium. Studies have revealed that SPI-1 mutants affect the colonization of chicken ceca [87, 88], while one report suggests that this effect is age dependent [89]. Further, it has been shown that the absence of SPI-1 can affect levels of *Salmonella* in internal organs [89, 90]. The major findings from these studies are discussed in sections 3.1 and 3.4.

1.3 *Salmonella* Pathogenicity Island-2 Type 3 Secretion System

The SPI-2 T3SS is located between centisome 30 and 31 of the *Salmonella* chromosome and is 40 kb in size (Figure 1.5). It encodes approximately 31 genes that are organized in two operons. One operon encodes genes of the secretion system apparatus (*ssa*), secretion system effectors (*sse*) and secretion system chaperones (*ssc*), while the other operon encodes the SsrAB two-component secretion system regulator (*ssr*) [91]. The SPI-2 secretion system plays a major role in intracellular survival and replication [92].

1.3.1 Structure

The surface structure of the SPI-2 apparatus (Figure 1.6) consists of a translocon, filament and needle. SseC and SseD form the translocon at the tip of the T3SS apparatus, while SseB forms a filament-like structure which connects the translocon to the needle protein, SsaG. The basal structure is composed of SsaC (outer membrane protein), SsaJ (periplasmic protein) and a complex of proteins that consist of the inner membrane and the export apparatus (SsaD, SsaR, SsaS, SsaT, SsaU and SsaV). The SPI-2 apparatus also contains an "ATPase" that provides the energy required for the secretion of effector proteins through this structure. The major candidates for this function are SsaN, SsaK and SsaQ [91].

1.3.2 Secretion

Salmonella species secrete many effector proteins across the vacuolar membrane of the SCV. The secretion of these proteins is mainly triggered in response to the intracellular phagosomal environment encountered by *Salmonella* inside host cells [93]. However, expression of SPI-2 genes has recently been reported in the intestinal lumen of mice, suggesting that expression of these genes may occur prior to invasion of host cells [94]. To date, at least 19 effector proteins (table 1.2) have been identified that are secreted through the SPI-2 T3SS some of which are located on SPI-2, while others are located outside this pathogenicity island. These virulence factors are involved in SCV maturation, trafficking and positioning. In addition, the aforementioned proteins also modulate the inflammatory response associated with *Salmonella* infection [28].

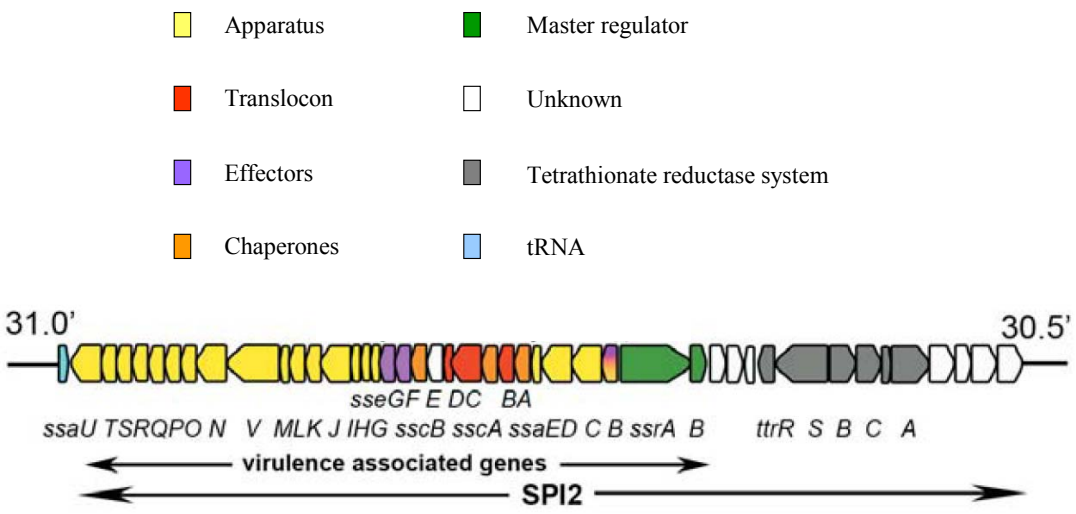


Figure 1.5. Schematic representation of the *S*. Enteritidis SPI-2 Type 3 Secretion System genes. Diagram was adapted and modified from [91].

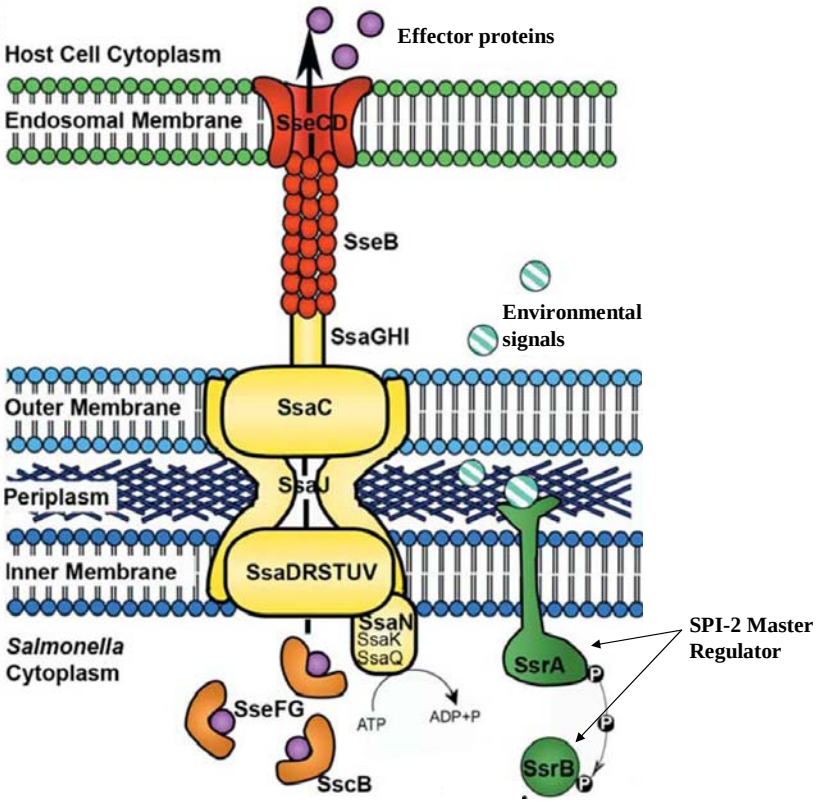


Figure 1.6. Structure of the *S.* Enteritidis SPI-2 Type 3 Secretion System. Diagram was adapted and modified from [91].

Table 1.2: List of effector proteins secreted by the *S*. Enteritidis SPI-2 T3SS. Table was adapted and modified from [28].

Effector protein	Gene location	Function	Reference
GogB	Bacteriophage Gifsy-1	Unknown	[95]
PipB	SPI-5	Associates with the SCV and sifs but is not required for intracellular survival or virulence in mice	[96]
PipB2	Outside SPI-2	Enhances growth of sifs	[97]
SifA	Outside SPI-2	SCV positioning, sif formation and re-directing traffic to SCV	[28, 98]
SifB	Outside SPI-2	Localizes with SCV and travels away from SCV along sifs	[99]
SopD2	Outside SPI-2	Localizes to the SCV membrane, involved in sif formation and is important for replication in macrophage and mice	[100, 101]
SpiC	SPI-2	Prevents fusion of late endosomes with SCV, required for translocation of SPI-2 proteins, important for survival in macrophages and virulence in mice	[102, 103]
SseF	SPI-2	Plays a role in Sif formation, re-directs traffic to SCV and maintains perinuclear position	[28, 98, 104]
SseG	SPI-2	Similar to SseF	
SseI	Bacteriophage Gifsy-1	Involved in the formation of actin surrounding the SCV	[105]
SseJ	Outside SPI-2	Maintains the integrity of the SCV membrane	[61, 106]
SseK1	Outside SPI-2	Localize to the host cytoplasm and has not been associated with virulence in mice	[107]
SseK2	Outside SPI-2	Similar to SseK1	[107]
SseK3	Bacteriophage	Unknown	[107]
SseL	Outside SPI-2	Suppresses NF-κB activity and causes cell death	[108, 109]
SspH2	Bacteriophage	Involved in the formation of actin surrounding the SCV	[105]
SteC	Outside SPI-2	Involved in the formation of actin surrounding the SCV	[110]
SpvB	*spv* plasmid	Inhibits SCV associated actin polymerization	[105]
SpvC	*spv* plasmid	Down regulates the inflammatory response	[111, 112]

The SPI-2 effector proteins SpiC, SseJ, SteC, SseI and SspH2 are involved in SCV maturation and trafficking. SpiC prevents the fusion of late endosomes with the SCV, which assists in delaying fusion of the SCV and lysosomes, allowing *Salmonella* to persist and replicate [103, 113]. The SPI-2 effector, SseJ, has deacylase activity and is involved in maintaining the integrity of the SCV membrane [61, 106]. SteC, SseI and SspH2 all play a role in the formation of a filamentous-actin (F-actin) meshwork surrounding the SCV [105, 110].

As the SCV continues to mature, it moves towards the nucleus (perinuclear position). The SPI-2 effector, SifA, is involved in maintaining the position of the SCV. The aforementioned protein, as well as SseF and SseG, are important for the formation of *Salmonella* induced filaments (sifs) [28], which are tube-like membranes that contain aggregates of lysosomal glycoproteins [98]. The growth of the Sifs are enhanced by the SPI-2 effector protein, PipB2 [97]. In addition, SifA, along with SseF and SseG, re-directs transport of exocytic vesicles to the SCV, a process which allows *Salmonella* to gain access to nutrients for bacterial replication and for the growing SCV [98].

Effector proteins secreted by the SPI-2 secretion system also play a role in down regulating the inflammatory response associated with *Salmonella* infections and have been linked with causing cell death. The SPI-2 effector, SseL, suppresses NF-κB activation by inhibiting IκBα ubiquitination, as a result of its deubiquitinase activity. This prevents IκBα degradation and consequently, IκBα suppresses NF-κB activity leading to lower levels of inflammation [108]. This effector protein (SseL) has also been shown to cause cell death. *Salmonella* Typhimurium mutant strains (*sseL*$^-$) have been shown to cause significantly less cytotoxicity in macrophages and have been shown to be attenuated for virulence in mice, relative to the wild type strain [109].

In summary, the main functions of SPI-2 effector proteins are SCV maturation and positioning, re-directing SCV traffic and modulating the inflammatory response (Figure 1.7).

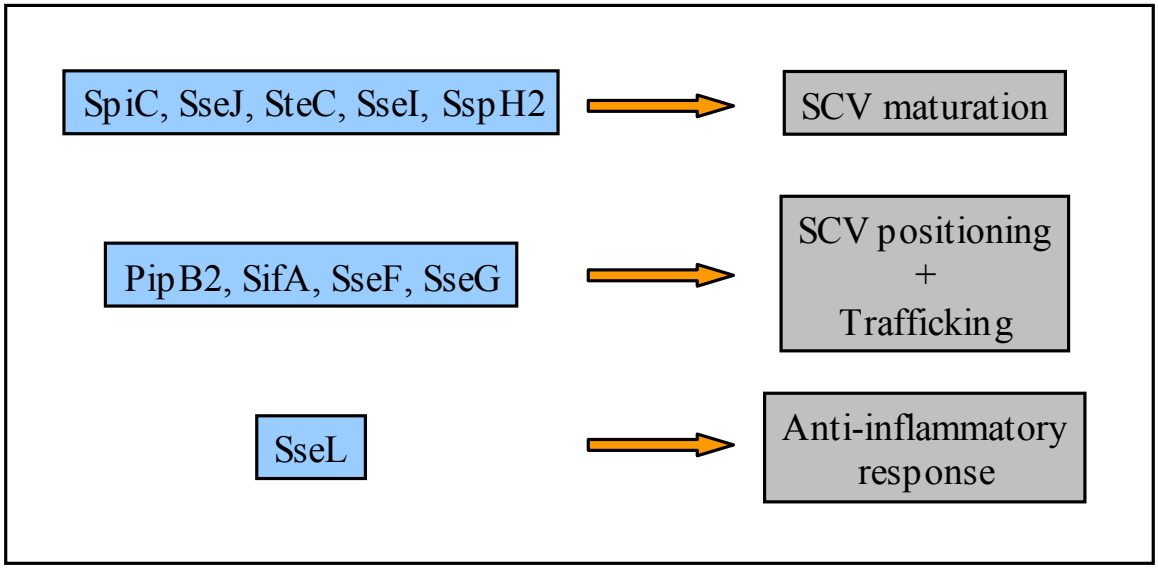


Figure 1.7. Summary of the main functions of *S*. Enteritidis SPI-2 effector proteins.

1.3.3 Regulation

The regulation of SPI-2 T3SS genes is complex and involves different systems. The SsrA/SsrB two component system is the master regulator of SPI-2 expression that is encoded on SPI-2. SsrA is a sensor kinase that, in response to environmental stimuli, phosphorylates the response regulator SsrB, which activates the expression of SPI-2 T3SS genes encoded on SPI-2 and outside the pathogenicity island [114-116].

The SsrA/SsrB two-component system is further regulated by the OmpR/EnvZ (described earlier in section 1.2.4) and PhoP/PhoQ two-component systems [116]. When *Salmonella* is grown under SPI-2 inducing conditions, EnvZ (sensor kinase) phosphorylates OmpR (response regulator) which in turn binds to the promoters of the SsrA/SsrB two component system, leading to activation of SPI-2 gene expression [117, 118]. Similarly, in conditions of low magnesium and low pH (conditions resembling the SCV), PhoQ (inner membrane sensor kinase) phosphorylates PhoP (cytoplasmic response regulator) which then activates the expression of the SsrA/SsrB system [119, 120].

The transcription of SPI-2 T3SS genes is also controlled by a family of proteins known as nucleoid associated proteins (NAPs) [116]. These proteins bind to bacterial chromosomal DNA and thus play an important role in the organization and compaction of bacterial chromatin. Hence, the NAPs are able to repress or activate the transcription of certain genes depending on different cellular requirements [121]. H-NS, YgdT and Hha are three NAPs that repress the transcription of SPI-2 genes [116]. On the other hand, integration host factor (IHF) and inversion stimulation (Fis) are two NAPs that are required for complete expression of SPI-2 genes. IHF is important for DNA bending and compaction, while Fis directly activates the transcription of SPI-2 genes by binding to the promoters of *spiR* and *ssaG* genes [122-124].

1.3.4 Role in virulence

The role of the SPI-2 T3SS has been studied extensively in mice, since they develop a systemic disease (explained in section 1.2.5). Using *S*. Typhimurium SPI-2 mutants it has been demonstrated that these strains are highly attenuated in mice relative

to the wild type strain. This corresponds to lower levels of the mutant strains in the internal organs of infected mice, suggesting that these strains were impaired in their ability to survive and replicate in host organs [125, 126]. Moreover, recent experiments have illustrated that the SPI-2 T3SS contributes to the intestinal form of disease in the murine and bovine models of infection [127, 128]. This finding implies that the role of this secretion system may not be exclusive to systemic disease during non-host adapted *Salmonella* pathogenesis. The role of the SPI-2 secretion system has also been evaluated in chickens, but this has not been extensively studied relative to the studies in the mouse and bovine models. Three studies performed in chickens indicated that lower levels of SPI-2 mutants (non-host adapted *Salmonella* species) were recovered from the internal organs of chickens relative to the wild type strain, implying that the SPI-2 T3SS is an important virulence factor in chickens [89, 129, 130].

1.4 *Salmonella* Enteritidis in Poultry

1.4.1 Sources of infection

Poultry flocks become infected with *S.* Enteritidis from a variety of sources. Incubation of *Salmonella* contaminated eggs with *Salmonella* free eggs at the hatchery results in high numbers of *Salmonella* positive chicks, suggesting that the hatchery is an important source of *Salmonella* infection. Similarly, poultry can also become infected by the transmission of this pathogen via aerosols from infected birds. The litter in poultry barns is another source of contamination since chicks contaminate the litter with fecal content containing *Salmonella*. Hence, other chickens easily become infected with *Salmonella* when they pick on fecal droppings from their littermates. Poultry flocks can also become contaminated with *Salmonella* after consumption of water. This happens when the chicks are young and their drinkers are located close to the ground. The chicks often defecate in the drinkers resulting in the contamination of the water and spread of the organism to the rest of the flock. Rodents also form a major source of *Salmonella* infection in poultry houses. They often shed high levels of *Salmonella* species in their feces [131]. In addition to the aforementioned sources, the presence of insects on poultry houses can significantly contribute to the dissemination of *Salmonella* species in poultry

flocks. These include cockroaches, lesser mealworms, flies and darkling beetles [132-135].

1.4.2 Pathogenesis

Chickens that come in contact with *S*. Enteritidis mostly develop gastrointestinal colonization, which results in shedding of the bacterium through the fecal contents. The bacteria may also cause a systemic infection leading to the spread of the organism to the internal organs (liver and spleen) [1, 131]. This occurs when *S*. Enteritidis breaches the intestinal epithelial surface via enterocytes, M cells or dendritic cells [136]. Once the bacteria reach the lamina propria they are internalized by macrophages and rapidly disseminate to the liver, spleen and other parts of the body through the thoracic duct [1, 6]. Systemic infection may also lead to infection of chicken ovaries resulting in the production of contaminated eggs. Young chicks (first few days of life) are more susceptible to colonization by *S*. Enteritidis relative to older birds and younger birds are more likely to develop a systemic infection. On rare occasions, young chicks can develop clinical signs that include progressive somnolence, drooping wings, ruffled feathers, anorexia, and diarrohea. In addition, chickens infected with other infectious agents like *Eimeria tenella* or immunosuppressive pathogens become more susceptible to infection by *Salmonella* species. The detection of *S.* Enteritidis in poultry flocks is often difficult because *S*. Enteritidis colonization is mainly asymptomatic [1, 131].

1.4.3 Immune response to *S*. Enteritidis

1.4.3.1 Innate immune response

The innate immune system is the first line of defense encountered by *S*. Enteritidis during the process of infection. It is composed of several components including physical barriers (e.g. epithelial surfaces), antimicrobial chemical components, complement system and specialized cells (e.g. natural killer cells, macrophages, dendritic cells and heterophils) [137].

Investigation of the dynamics of immune cell filtration in day old chicks after infection with *S*. Enteritidis has revealed that heterophils were the first group of cells that infiltrated the cecal lamina propria at 12 hours post infection, followed by macrophages and T-lymphocytes at 20 hours post infection, and B cells at 24 hours post infection [138, 139]. This suggested that the chicken innate immune system was important in *Salmonella* infection. This notion was supported by a study in which heterophil depleted chickens were more susceptible to *S*. Enteritidis organ invasion [140, 141]. Moreover, recently it has been demonstrated that chicken heterophils release Cathelicidin-2 (antimicrobial peptide) upon contact with *Salmonella* which has potent bactericidal activity [142]. Cathelicidin-2 also has the capacity to bind to LPS in order to block LPS mediated proinflammatory gene expression [137].

Another important component of the chicken innate immune system are toll-like receptors (TLRs) which are composed of transmembrane sensor proteins that recognize pathogen associated molecular patterns (PAMPs) leading to the induction of innate immunity [143]. Chicken TLR-4 plays an important role during the host response to *Salmonella* infection. This receptor recognises *Salmonella* LPS leading to the activation of heterophils and an increase in the secretion of pro-inflammatory cytokines IL-1β, IL-6 and IL-18 [144, 145]. In the same way, chicken TLR-5 recognizes *Salmonella* flagella leading to an upregulation of IL-1β (causing inflammation) *in vitro* and in chickens. Interestingly, aflagellar *S*. Typhimurium does not upregulate IL-1β but is isolated at higher levels from the internal organs of chickens infected with the aflagellar mutant strain relative to the wild type strain. This suggests that the absence of the flagella allows *S*. Typhimurium to evade recognition by the host immune system [143, 146].

Similarly, other experiments have revealed that oral inoculation of chickens with non-host adapted *Salmonella* species (*S*. Enteritidis or *S*. Typhimurium) resulted in the up-regulation of IL-1β, IL-18 and TGF-β4 mRNA levels (as determined by real-time reverse transcription (RT)-PCR) in the spleens of infected birds, while the level of IL-4 was down-regulated [147-150]. In the ceca of infected chickens, it has been observed that the mRNA levels of IL-1β, IL-6, IL-8, IL-12, IL-18 and TGF-β4 have been upregulated

relative to non-infected control birds [149-152]. The cytokines IL-1β, IL-6 and IL-8 are pro-inflammatory cytokines that lead to the recruitment of immune cells leading to inflammation in the host tissue. On the other hand, the increase in the levels of IL-12 and IL-18, in response to *Salmonella* infection, is indicative of a cellular immune response [153].

1.4.3.2 Adaptive immune response

Chickens infected orally with *S.* Typhimurium have been associated with higher levels of interferon-gamma (IFNγ) in splenocytes relative to control birds. In addition, levels of IFNγ gradually declined as the infection started to clear, suggesting that IFNγ (a strong indicator of cell mediated immunity) played an important role in clearance of *S.* Typhimurium infection [147]. As well, it has been demonstrated that intraperitoneal administration of IFNγ to chickens results in lower organ colonization following *S.* Typhimurium challenge [154]. Similarly, another group has shown that chickens infected with *S*. Typhimurium developed strong T cell responses (as measured by proliferation assays) relative to control birds and this also correlated with clearance of the infection [150]. In a more detailed study, the authors characterized the T cell composition of lymphocytes in the blood as well as in different organs following *S*. Typhimurium infection. $CD8^+$ T cells were upregulated in the blood, ceca, bursa of fabricius and the spleen relative to the non-infected birds [155]. This provided further support to imply that cell mediated immunity was an important component in the defense against *S*. Typhimurium infections.

Infection of chickens with *S*. Typhimurium has often been associated with significant serum IgM, IgG (IgY) and IgA antibody titers 5-7 days post-infection. However, despite having high antibody titers, this has not always resulted in lower levels of *Salmonella* or clearance of the organism [156]. To examine the role of antibodies in *Salmonella* infections, B cell depleted chickens have been used. Chickens that were chemically bursectomized (using cyclophosphamide or testosterone proprionate) have been shown to have higher levels of *S.* Enteritidis in cecal contents relative to healthy chickens, but this difference was not observed in internal organs. It was suggested that

this may indicate that secretory IgA plays a role in clearance of *Salmonella* infections from the chicken gut [157]. However, recently, it has been demonstrated that surgically bursectomized chickens had levels of *Salmonella* that were identical to healthy chickens in cecal contents as well as in internal organs. Moreover, the chickens were able to clear the infection at the same rate as healthy chickens [158]. This study suggests that the humoral response may not be critical for defense against *Salmonella* infections in chickens. Additionally, a recent report has indicated that using chemical methods to deplete B cells in chickens also affects other cells [156], adding further support to the findings in the study which used surgically bursectomized chickens [158]. Taken together, these studies imply that the humoral response to *Salmonella* infection may not be crucial in the clearance of infection. However, the role of antibodies has not been excluded in chickens since further work needs to be done to investigate the dynamics of the immune response to *Salmonella* infection [156].

1.5 Control of *Salmonella* Enteritidis

1.5.1 Management practices

Good flock management and sanitation practices are important since *S*. Enteritidis can be introduced into poultry houses from many different sources as discussed in section 1.4.1. One of the most important aspects of poultry house management is to ensure that eggs or chicks coming into the facility are from a breeding flock that has been extensively tested for *Salmonella* species and is proven to be *Salmonella* free. Secondly, eggs that are set for hatching should be properly disinfected and hatched under strict conditions to minimize the chances of *Salmonella* contamination. In addition, between flocks, the poultry house should be cleaned and disinfected using an effective method since not all methods are effective in eliminating *S*. Enteritidis [159, 160]. Formaldehyde fumigation has been shown to highly effective and should be used to disinfect poultry facilities [161]. Fourthly, since rodents and insects are important vectors of *Salmonella* transmission in poultry houses (discussed in section 1.4.1), strict control of the aforementioned is critical given that a single mouse dropping can contain 10^5 *S*. Enteritidis cells [162]. Moreover, a study has shown that the levels of *S*. Enteritidis in

mice from a poultry house positive for this pathogen were four times higher than in mice from a facility negative for *S*. Enteritidis [163]. Biosecurity is also an important component of good management practices at poultry houses. This includes ensuring that all personnel entering the facility either change their footware or use the boot dip disinfection method. Additionally, equipment and machinery that either enters or that is in use at the facility should be cleaned and disinfected [160, 164]. The feed that is used for chickens at the poultry houses should not contain any animal proteins and should be *Salmonella* free [160]. This can be achieved by the addition of chemicals (antimicrobials) in the feed, using heat or irradiation [164]. Another important component of good management practices includes a *Salmonella* free water supply. This involves ensuring that the water supplied to the facility has been treated properly since water contaminated with *S*. Enteritidis has been associated with *S*. Enteritidis contamination of a laying flock [165]. Other factors that involve good management at facilities housing poultry flocks are controlling moisture levels and ensuring that there is adequate ventilation [160].

1.5.2 Bacteriophages

Bacteriophage therapy in chickens involves the use of phages that are orally administered to chickens a few days after oral challenge with non-host specific *Salmonella* species (e.g. *S*. Enteritidis and *S*. Typhimurium). These phages recognize specific receptors on the surface of bacteria and inject their DNA into the bacterial cell. Using the cell's machinery, the phage replicates and assembles virulent phages which then lyse the bacterial cell in order to infect other cells [166].

A recent study evaluated the efficacy of broad host range phages against *S*. Enteritidis. Chickens were treated orally with either 10^9 plaque forming units (pfu) or 10^{11} pfu of phages two days after oral challenge with *S*. Enteritidis. At the higher dose, the levels of *S*. Enteritidis were significantly lower in the cecal contents (about 2 to 4 log units lower) relative to the control group. However, the study did not examine the effect of phage therapy on systemic levels of *S*. Enteritidis [167]. On the other hand, results from other studies have given variable results suggesting that phage therapy may have potential as a pre-harvest intervention measure and that more research needs to be

performed to investigate the effects of phage therapy on *S*. Enteritidis colonization in chickens [166].

1.5.3 Competitive Exclusion

Competitive exclusion, in the context of poultry, refers to the administration of normal bacterial flora from the gastrointestinal tract of poultry to reduce colonization by enteric pathogens [168]. This inhibition effect is thought to occur by either interference with the attachment of the enteric pathogen to the host intestinal wall or inhibition of the growth of the pathogen due to a change in the intestinal environment. In the case of *Salmonella* species, competitive exclusion products from older chickens have been given orally to younger birds leading to reduced levels of *Salmonella* species in the gastrointestinal tract of these pathogens [160, 168]. The reduction in the levels of this pathogen has also been associated with lower numbers of carcass contamination [169]. Hence, the use of competitive exclusion products is an attractive strategy that can be used as one of the components to form part of a comprehensive *Salmonella* control program. However, the use of this approach also has certain limitations. First of all, many of these products are composed of undefined cultures. Thus, this involves introducing undefined live organisms into chickens and the poultry houses. Additionally, it may also lead to the introduction of antibiotic resistant bacteria and possibly result in the transfer of antibiotic resistance to other non-resistant bacterial strains. Nonetheless, attempts have been made to define competitive exclusion cultures and possible candidates responsible for the protective effect have been suggested that include *Lactobacillus* species, *Bifidobacterium* species, *Escherichia coli*, *Bacillus subtilis* and *Streptococcus cristatus*. Secondly, the protective effect of these cultures has been observed in younger birds which have not yet developed a mature intestinal microflora. Thus, the use of these products is not a reliable option for older birds. Thirdly, this treatment is not very effective in chickens that have an altered microflora due to antibiotic treatment or chickens that have been subject to feed or water withdrawal [160].

1.5.4 Vaccination

Vaccination of chickens for protection against non-host specific *Salmonella* serotypes has been widely used as an intervention strategy to reduce the prevalence of *Salmonella* in poultry flocks in order to protect consumers from food-borne disease transmission [160, 164, 170, 171]. This strategy aims to utilize the host's immune system to decrease the levels of the pathogen that are associated with the animal upon infection. Moreover, extensive vaccination of chickens in the United Kingdom since 1997 (Figure 1.8) has successfully reduced the incidence of human *S*. Enteritidis infections [10]. In the same way, vaccination along with other intervention strategies has drastically lowered the number of human *S*. Enteritidis infections in Belgium [172]. The *Salmonella* vaccines that have been used to date are generally classified into three categories: Live attenuated strains, killed *Salmonella* and subunit vaccines.

1.5.4.1 Live attenuated vaccines

Live attenuated strains of *Salmonella* are constructed mainly by the mutation or deletion of genes that are important for virulence, metabolism or survival. These strains are associated with several advantages. They can be administered orally and to young chicks, induce rapid protection, and activate both humoral and cell-mediated responses. However, the main concern with live attenuated strains is that they consist of a living organism which may persist for extended periods in the chickens as well as their environment, posing a threat to human health [170].

S. Enteritidis *aroA* mutants (strains impaired in the ability to synthesize aromatic compounds) have been tested as live vaccine candidates chickens. Oral vaccination with the aforementioned strain resulted in a reduction of the levels of *S*. Enteritidis in the livers, spleens, cecal contents and ovaries relative to the non-vaccinated group [173]. Similarly, a *cya-crp S*. Typhimurium double mutant (deletion in the genes encoding adenylate cyclase and cyclic adenosine monophosphate receptor protein) used for vaccinating chickens orally (10^8 cfu) at 1 and 14 days of age greatly reduced the levels of the challenge strain (10^6 cfu *S*. Typhimurium) in the ceca (approximately 6 log units) relative to the control group. As well, the levels of the challenge strain in the spleen were

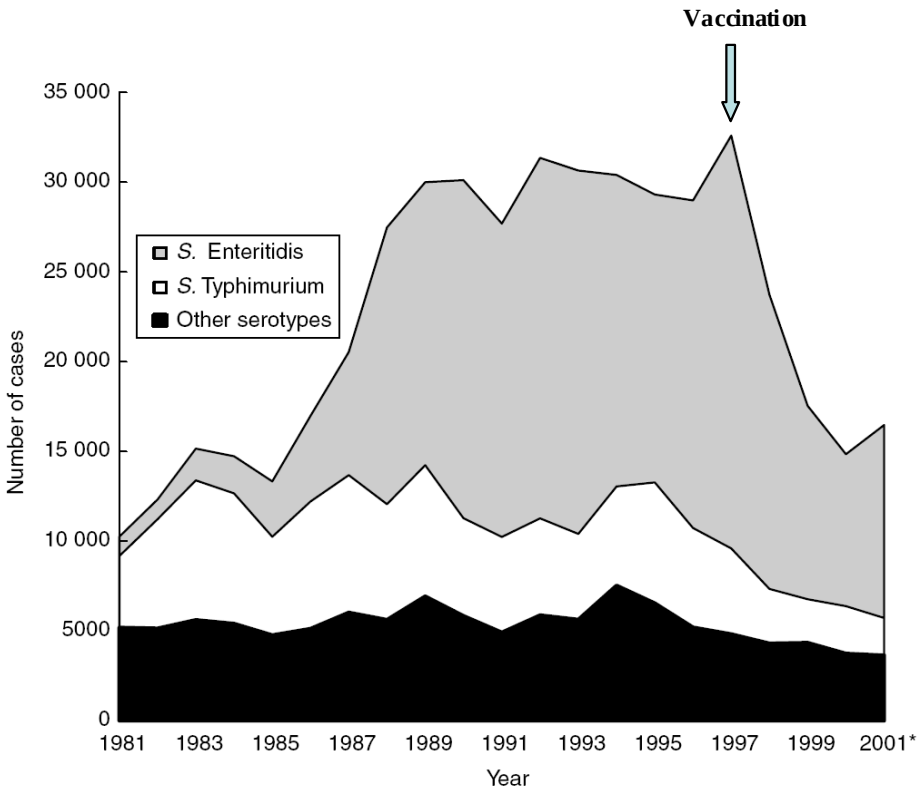


Figure 1.8. Human *Salmonella* cases in the United Kingdom from 1981 – 2001. Diagram was adapted and modified from [10].

not detectable in the vaccinated group. However, when the same vaccine was used for protection against *S*. Enteritidis oral challenge, the levels of the challenge strain in the ceca were only slightly lower relative to the control group, while the levels in the spleen were not detectable in the vaccinated group [174]. A temperature sensitive *S*. Enteritidis mutant (able to grow at 28°C and not at 37°C) has also been evaluated as a live attenuated vaccine strain in chickens. Chickens were immunized at 1 and 14 days of age orally or intra-peritoneally with 10^{10} cfu of the mutant strain and were challenged (orally) at 28 days of age with 10^{8} cfu of virulent *S*. Enteritidis. All vaccinated groups demonstrated lower levels (several log units lower) of the challenge strain in the ceca and spleens compared to the control group [175].

The *Salmonella enterica* subspecies *enterica* serovar Gallinarum (*S*. Gallinarum) 9R strain (modification in the LPS) is another live attenuated strain that has been widely used in chickens for protection against *S*. Enteritidis. This strain has been tested under field conditions where chickens were vaccinated subcutaneously at 6 weeks, followed by a booster injection at 14-16 weeks of age as per the recommendation of the commercial vaccine (Nobilis SG 9R). Chickens were monitored for *S*. Enteritidis shedding prior to the 1st vaccination and up to 6 weeks after the 1st vaccination. The results indicated that 2.5 % of the flocks were positive for *S*. Enteritidis in the vaccinated groups relative to the control group (11.5 %) [176]. TAD *Salmonella* vac® E is a *S*. Enteritidis live vaccine constructed by chemical mutagenesis which has prolonged generation times and is less virulent. To evaluate the efficacy of this strain, chickens were vaccinated orally with 10^{8} cfu of TAD *Salmonella* vac® E at 1 day of age, followed by two boosters at 6 and 16 weeks of age. The chickens were intravenously challenged at 24 weeks of age with 10^{7} cfu of *S*. Enteritidis. Vaccinated chickens had significantly lower numbers of positive livers (12/28) and oviducts (6/28) relative to the non-vaccinated chickens (23/30 livers and 15/29 oviducts). Similarly, eggs obtained from vaccinated chickens had fewer positive samples (9/35) compared to the control group (15/35) at 22 weeks of age [177].

In the aforementioned studies, different live attenuated *Salmonella* strains have been used for evaluating the efficacy of each strain to confer protection against

Salmonella challenge. However, in most of the experiments, the oral challenge was conducted shortly after immunization with the vaccine strain. Hence, it is possible that the vaccination strain may have colonization-inhibition effects that lead to reduced colonization and systemic spread of the challenge strain [170]. Moreover, not all the live attenuated strains (described above) have been tested under field conditions. In addition, these vaccine strains have not been very effective in inducing cross-protection against other non-host adapted serovars [160].

1.5.4.2 Killed vaccines

Killed *Salmonella* vaccines consist of whole cell bacteria that are inactivated using either heat, formalin or acetone. Most of these strains are delivered subcutaneously and require at least two injections. A major advantage conferred by this class of vaccines is that they do not contain a live organism that could potentially survive in the environment and affect human health. However, the use of these vaccines has been mainly associated with strong antibody responses [160, 170].

Salenvac® is a commercially available killed vaccine licensed for use in the United Kingdom. It consists of an inactivated *S.* Enteritidis strain grown under iron limiting conditions (growth under iron limiting conditions is thought to stimulate the production of virulence factors in order to mimic the host environment). The efficacy of Salenvac® was evaluated in a study in which laying chickens were vaccinated subcutaneously at day 1 and 4 weeks of age (group 1), while the second group followed the same regime with a third immunization at 8 weeks of age (group 2). The chickens were challenged intravenously at different ages (8, 17, 23, 30 or 59 weeks) with 10^7 cfu of *S*. Enteritidis. Vaccinated birds had significantly lower *S*. Enteritidis fecal shedding, organ colonization and egg contamination (shells and internal contents) relative to the control birds [178]. It is obvious that the Salenvac® vaccine is highly effective in protecting laying chickens against a high dose of *S*. Enteritidis challenge. However, this study would have been more relevant if an oral challenge had been given since that would be closer to the real situation.

Layermune SE® is a killed *S*. Enteritidis vaccine that is commercially available. It is composed of multiple strains of *S*. Enteritidis formulated in an oil based adjuvant. This vaccine was tested in a study in which layers and broilers were vaccinated subcutaneously at five and nine weeks of age. The chickens were orally challenged at ten weeks of age with 10^8 cfu of *S*. Enteritidis. The vaccine reduced the levels of *S*. Enteritidis in the ceca, livers and spleens of the vaccinated group by several log units relative to the control group in layers but was not very effective in broiler chickens [179].

Similarly, Poulvac SE®, a commercially available killed *S*. Enteritidis vaccine (contains *S*. Enteritidis phage types 4, 8 and 13a), has been evaluated for protection in progeny derived from broiler breeders vaccinated with this strain. Broilers were vaccinated subcutaneously at twelve and twenty weeks of age. Progeny derived from these hens were orally challenged at 1 day or two weeks of age with 10^5 cfu or 10^9 cfu of *S*. Enteritidis, respectively. The results indicated that the number of birds positive for *S*. Enteritidis were much lower in the progeny derived from the vaccinated group (7/25) challenged at 1 day of age compared to the control group (25/25). However, the vaccine was not very effective in progeny challenged at two weeks of age relative to the control group. The vaccine did not have a major effect on the levels of *S*. Enteritidis (based on direct plating) in the livers, spleens and ceca of progeny obtained from the vaccinated chickens that were challenged at 1 day or at two weeks of age [180]. Another study evaluated the efficacy of a *S*. Enteritidis killed strain. In this study killed *S*. Enteritidis was formulated with microspheres and used to vaccinate chickens orally or intramuscularly at two weeks of age. The chickens were orally challenged at six weeks of age with 10^9 cfu of *S*. Enteritidis. Vaccination reduced the number of organs (ceca, liver and spleen) that were positive for *S*. Enteritidis (18-26 %) relative to the control group (44 %) [181].

1.5.4.3 Subunit vaccines

Subunit vaccines consist of a single protein or a mixture of proteins that are usually expressed on the surface of the cell and are considered to be important in

virulence. Such proteins are mainly delivered subcutaneously, unless they are formulated appropriately for oral delivery.

A vaccine containing an extract of *S*. Enteritidis outer membrane proteins was evaluated in chickens. The chickens were immunized orally or subcutaneously at 8 weeks of age, followed by a booster at 11 weeks of age. At thirteen weeks of age, the chickens were orally challenged with 10^7 cfu of *S*. Enteritidis. The numbers of chickens positive for *S*. Enteritidis, as determined by cloacal swabbing, were mostly negative in the group vaccinated subcutaneously relative to the control group (100% positive). However, this study did not examine the levels of *S*. Enteritidis present in the liver, spleen or ceca [182]. In the same way, another group evaluated the effect of immunizing chickens with *S*. Enteritidis outer membrane proteins. The chickens were immunized subcutaneously at nine and eleven weeks of age. At twelve weeks of age, the chickens were orally challenged with 10^8 cfu of *S*. Enteritidis. Vaccinated chickens had lower levels of *S*. Enteritidis (2-3 log units) in the ceca and small intestine relative to the control group. However, the levels of the challenge strain were not determined in the internal organs [183].

S. Enteritidis type 1 fimbriae have been evaluated as potential subunit vaccine candidates. Chickens were vaccinated subcutaneously at 18 weeks of age with purified type 1 fimbriae, followed by a booster injection at 21 weeks of age. At 24 weeks of age, the birds were challenged intravenously with 10^7 cfu of *S*. Enteritidis. The results suggested that the vaccine did not have an effect on the levels of *S*. Enteritidis in the ceca, livers or spleens. However, there was a mild reduction in the number of egg shells and oviducts contaminated with *S*. Enteritidis in the vaccinated group compared to the non-vaccinated control [184]. In another study, a peptide containing part of the FliC protein (forms the flagellar filament) was used to immunize chickens subcutaneously at 3 and 5 week of age, with an oral challenge of 10^7 cfu of *S*. Enteritidis at 7 weeks of age. The FliC based vaccine significantly reduced (2 log units) the levels of the challenge strain at 3 weeks post challenge in the cecal contents based on direct plating relative to the control group. The vaccine did not affect the levels of the challenge strain in the livers and spleens of the vaccinated birds [185].

Taken together, it can be concluded that vaccination of chickens with either live attenuated, killed or subunit vaccines has resulted in lowering the levels of *S*. Enteritidis (summarized in Table 1.3). Based on the data presented from different vaccine efficacy studies, it is difficult to infer that one class of vaccines confers greater protection than the other, although few studies have examined the efficacy of subunit vaccines. Recently, one study examined the efficacy of four commercially available *Salmonella* vaccines (3 live attenuated strains and 1 killed strain) in the U.K. in reducing *S*. Enteritidis colonization of laying hens. Chickens vaccinated with the killed strain had significantly lower levels of *S*. Enteritidis relative to the other groups, suggesting that killed *Salmonella* strains may be more effective in controlling the prevalence of *Salmonella* in poultry [186]. Moreover, killed *Salmonella* strains and subunit vaccines have an advantage over live attenuated strains since the latter are composed of a living organism. With the continued prevalence of *S*. Enteritidis human infections, there is still a need for improved and well defined vaccines to control the spread of this bacterium along with other intervention strategies [170].

1.5.5 Consumer Education

Since the aim of *Salmonella* control programs is to ultimately reduce the number of human *Salmonella* infections, it is crucial that consumers are also informed about strategies that can be applied in their daily lives to lower their chances of becoming infected with this pathogen. Consumption of undercooked poultry meat and eggs is a significant pathway for human exposure not only to *Salmonella* species, but also to other pathogens such as *Campylobacter* species. Therefore, informing consumers (through different educational tools) to thoroughly cook all foods that contain poultry meat or eggs is an important step in risk reduction. As well, labeling poultry meat and eggs or products containing the aforementioned will remind consumers to be cautious and follow procedures. Similarly, it has been determined that cross-contamination of food products with *Salmonella* species while handling poultry meat or eggs is also a major source of human exposure to this pathogen. Hence, consumers must be advised to use either separate cutting boards and utensils in the kitchen when handling poultry products or to properly wash and clean them before handling other food products [187].

Table 1.3 Summary of major studies carried out to evaluate the efficacy of *Salmonella* vaccines in protection against *S*. Enteritidis challenge (for details please see text).

Vaccine (type)	Route of delivery	Frequency of vaccination	Bacterial challenge	Results of vaccinated group
S. Enteritidis Δ*aroA* (live)	Oral 10^5 or 10^9 cfu	1 day of age	10^8 cfu *S*. Enteritidis (oral) at 8 wks	Levels of challenge strain were 1-2 log units lower in the liver and spleen and much lower in the ceca at 1 and 4 days postchallenge.
S. Typhimurium Δ*cya-crp* (live)	Oral 10^8 cfu	1 and 14 days of age	10^6 cfu *S*. Enteritidis (oral) at 4 wks	Challenge strain was not detectable in the spleen and levels in the ceca were similar to the control at 2 weeks postchallenge.
Ts *S*. Enteritidis mutant (live)	Oral 10^{10} cfu and/or intraperitoneal 10^8 cfu	1 and 14 days of age	10^9 cfu *S*. Enteritidis (oral) at 4 wks	Challenge strain was 2-5 log units lower in the spleen and 5 log units lower in the ceca upto 21 days postchallenge in orally vaccinated group.
S. Gallinarum 9R Nobilis® (live)	Subcutaneous	6 weeks and 14-16 weeks	Field conditions	2.5 % of the vaccinated flocks were positive for *Salmonella* relative to the control group (11.5%) as determined by fecal swabs.
TAD *Salmonella* vac® E (live)	Oral 10^8 cfu	1 day of age, 6 weeks and 16 weeks	10^7 cfu *S*. Enteritidis (i.v.) at 24 weeks	12/28 livers and 6/28 oviducts were positive for *Salmonella* relative to the control, 23/30 and 15/29, respectively, at 3 weeks postchallenge.
Salenvac® (killed)	Subcutaneous	Day 1 and 4 weeks	10^7 cfu *S*. Enteritidis (i.v.) at 17, 23, 30 and 59 wks	22.6% of cloacal swabs and 12.5% internal organs were positive for *Salmonella* over 21 days postchallenge compared to the control (48.0% and 28.0%, respectively).

Vaccine (type)	Route of delivery	Frequency of vaccination	Bacterial challenge	Results of vaccinated group
Layermune SE® (killed)	Subcutaneous	5 and 9 weeks	10^8 cfu *S.* Enteritidis (oral)	Levels of challenge strain were 1-2 log units lower in liver, spleen and ceca of vaccinated layers but not broilers.
Poulvac SE® (killed)	Subcutaneous	12 and 20 weeks	10^5 cfu *S.* Enteritidis (oral) for day old progeny 10^9 cfu *S.* Enteritidis for two week old progeny	7/25 birds were positive for *Salmonella* relative to the control (25/25) in day old progeny, while 2/25 birds were positive relative to the control (9/25).
S. Enteritidis outer membrane protein extract	Oral or subcutaneous	8 and 11 weeks	10^7 cfu *S.* Enteritidis (oral) at 13 weeks	Very few chickens shed the challenge strain compared to the control (100%) up to 4 weeks postchallenge.
75.6 and 82.3 kDa *S.* Enteritidis outer membrane proteins	Subcutaneous	9 and 11 weeks	10^8 cfu *S.* Enteritidis (oral) at 12 weeks	Levels of the challenge strain were 2-3 log units lower in the ceca relative to the control.
S. Enteritidis Type I fimbriae	Subcutaneous	18 and 21 weeks	10^7 cfu *S.* Enteritidis (i.v.) at 24 weeks	Levels of the challenge strain were similar in the liver, spleen and ceca relative to the control upto 3 weeks postchallenge.
S. Enteritidis FliC protein	Subcutaneous	3 and 5 weeks	10^7 cfu *S.* Enteritidis (oral) at 7 weeks	Levels of the challenge strain were 3 log units lower in the cecal contents at 3 weeks postchallenge relative to the control.

1.5.6 Testing

In order to ensure that the above mentioned intervention strategies are successful in reducing the levels of *S*. Enteritidis, it is crucial that regular microbiological testing is carried out. This should include testing of chicken fecal shedding, internal organs, eggs and poultry facilities for the presence of *S*. Enteritidis [160]. As well, it is important that other post-slaughter intervention strategies are put in place that include microbiological testing for *Salmonella* species at slaughter houses and meat processing plants. This would be greatly enhanced by the adoption of Hazard Analysis Critical Control Point (HACCP) programs that have been developed by several government agencies like the Canadian Food Inspection Agency and the Food Safety and Inspection Service in the U.S. Department of Agriculture. These programs are specially designed to monitor, control and prevent food safety risks.

2.0 HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

S. Enteritidis SPI-1 is important for cecal colonization and systemic spread in chickens and vaccination with SPI-1 T3SS proteins can be used to reduce colonization and systemic spread of the organism.

2.2 Rationale

Salmonella Pathogenicity Island-1 has been shown to be an important virulence determinant in the bovine, murine and porcine models of infection. However, the role of the SPI-1 T3SS has not been well characterized in chickens. In addition, vaccination of chickens with live attenuated or killed *Salmonella* strains has resulted in the reduction of the prevalence of *Salmonella* Enteritidis in chickens. However, there are safety concerns associated with live attenuated strains and the protective antigens are not well defined in killed *Salmonella* strains. Based on this, we wanted to test the role of the *S*. Enteritidis SPI-1 T3SS in cecal colonization and systemic spread in chickens. Furthermore, we intended to identify T3SS proteins that could be used as vaccine candidates for protection against *S*. Enteritidis oral challenge.

2.3 Objectives

1. To construct *S*. Enteritidis SPI-1 mutants and test the role of SPI-1 in invasion *in vitro* using polarized Caco-2 cells and *in vivo* in chickens.
2. To determine if sera against SPI-1 T3SS proteins effectively inhibit invasion by *S*. Enteritidis *in vitro*.
3. To vaccinate chickens with SPI-1 T3SS proteins and test for protection against *S*. Enteritidis.

3.0 *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS PATHOGENICITY ISLAND-1 IS NOT ESSENTIAL FOR, BUT FACILITATES RAPID SYSTEMIC INFECTION IN CHICKENS

Published in Infection and Immunity (Desin *et al*, 2009) [188].

3.1 Introduction

Salmonella enterica are Gram negative enteropathogenic bacteria. Within the *S. enterica* species, more than 2300 serovars have been identified, of which serovars Enteritidis and Typhimurium have been the most frequently associated with human infections [5]. *S.* Enteritidis is a well known zoonotic pathogen [25] and infected poultry are among the most common reservoir of salmonellae that can be transmitted through the food chain to humans [1]. In young chicks, *S.* Enteritidis infection can lead to increased incidence of illness, while older birds are less susceptible to the effects of this pathogen often experiencing intestinal colonization and even systemic dissemination without significant morbidity or mortality. Hence, a better understanding of the pathogenesis of *S.* Enteritidis in chicken may subsequently lower the rates of human disease caused by this pathogen.

S. Enteritidis requires a substantial number of genes for virulence, which are clustered in large chromosomal regions known as *Salmonella* pathogenicity islands (SPI) [26]. Two of those pathogenicity islands encode two functionally distinct T3SS which are utilized as "molecular syringes" to translocate virulence determinants, called effector proteins, from the bacterial cytoplasm into [25, 189] or in the vicinity of the target cell [75]. Effector proteins delivered by the SPI-1 T3SS are mainly involved in host cell invasion by inducing membrane ruffling and disrupting actin polymerization to facilitate bacterial uptake [24]. The SPI-2 T3SS plays a major role in systemic virulence and in facilitating intracellular survival, especially within macrophages. However, recent evidence suggests that the SPI-2 T3SS also plays a role in intestinal colonization [127, 128] and is expressed prior to bacterial uptake [94].

Invasion of epithelial cells by *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) has been shown to disrupt tight junctions [190-192], which along with other components form important intercellular junctions found in polarized epithelial cells. Tight junctions regulate the paracellular flow of ions and solutes across the intestinal epithelium. They also maintain distinct apical and basolateral domains with well defined plasma membrane components [193]. SPI-1 effector proteins have been associated with the disruption of tight junctions [191, 192, 194] by activating Cdc42 and Rac-1 (Rho family GTPases), which subsequently activate signal transduction pathways that lead to the reorganization of actin, resulting in uptake of *Salmonella* [65]. Further, *S.* Typhimurium SPI-1 effectors SopB, SopE, SopE2 and SipA have been identified as major contributors in the disruption of tight junctions [194].

The contribution of *S.* Typhimurium SPI-1 to intestinal cell invasion has been studied in cell culture using different SPI-1 deletion mutants. Mutations in the SPI-1 regulatory gene *hilA* and *sipB* (SPI-1 effector gene) have been shown to be attenuated in invasion relative to the wild type strain in porcine intestinal epitheloid (IPI-21) cells. However, the use of polarized porcine intestinal epithelial cells (IPEC-J2) has revealed that in addition to *hilA* and *sipB* mutant strains, a *sipA* (SPI-1 effector gene) mutant strain was also recovered at a significantly lower rate as compared to the wild type strain [195], suggesting that SPI-1 is important for efficient invasion. In another study, it was demonstrated that *S.* Typhimurium SPI-1 effectors, SipA, SopA, SopB, SopD and SopE2 all contributed to invasion of polarized human colon carcinoma cells (T84). Mutants lacking the genes for the aforementioned effector proteins were less invasive relative to the wild type strain [196]. Moreover, an *S.* Typhimurium strain lacking the *invG* gene (encoding an outer membrane component of the SPI-1 T3SS apparatus) was also impaired in invasion of COS-7 cells [197]. Taken together, the results from these invasion studies suggest that SPI-1 plays a role in invasion in cell culture. However, most of the data available is from research that has been conducted using *S.* Typhimurium.

Several *in vivo* experiments have been performed to investigate the role of SPI-1 during the course of a *Salmonella* infection in different animal species using deletion

mutants in SPI-1 genes. In the murine model of infection, it was observed that *S.* Typhimurium strains containing mutations in *hilA* and *invG* were recovered from intestinal contents and systemic sites at a lower frequency than the wild type strain [198]. Other groups have reported that a functional SPI-1 T3SS is required to induce intestinal inflammation and cause significant histopathological changes in the streptomycin treated mouse model of infectious enterocolitis [128, 197, 199]. Similarly, in the bovine model of enteritis, SPI-1 has been shown to be important for intestinal colonization [200]. In addition, it has been demonstrated that *S.* Typhimurium mutants in SPI-1 genes (*sipA*, *sipB, hilA*), were impaired in their ability to colonize the porcine gut in a ligated intestinal loop model [195]. However, a recent study has shown that a SPI-1 functional mutant induces intestinal pathology that is very similar to the wild type strain when studied 5 days post challenge in a novel bovine ileal loop model [127].

Studies investigating the contribution of SPI-1 in chicken during the course of a *S.* Enteritidis infection are limited in number and have mostly observed colonization and systemic infection over a short time frame. Infection of one-day old birds with *S.* Enteritidis strains containing mutations in the *invA*, *invB* and *invC* genes (SPI-1 genes) have shown to be attenuated in the colonization of the gastrointestinal tract as well as in the systemic spread of the organism over a period of six days post-infection [87]. In another study, it was observed that *S.* Enteritidis *sipD* mutants were unable to colonize spleens as compared to the wild type strain three days post-infection in one day old chicks [90]. However, the colonization of the spleen was only measured at one time point, making it difficult to predict the effect of the mutant strain prior to and after, the third day following infection. A similar trend was seen in the ceca of one-day old chicks challenged with a *S.* Enteritidis *hilA* mutant strain over a period of twenty-eight days post-infection [88]. However, the *hilA* mutant strain did not have a significant impact on the infection of the livers and spleens. Recently, the impact of SPI-1 was examined using a *S.* Typhimurium *spaS* mutant strain in one-day old and one-week old birds [89]. Inactivation of *spaS* (SPI-1 structural protein) did not affect colonization of the liver or the ceca of 1-day old birds over a period of 72 hours post-infection. In 1-week old birds,

the same strain was recovered at lower levels from the ceca over a period of 14 days post-infection, while the recovery from the liver was lower at 3 days post-infection [89].

Taken together, the experimental evidence from the *in vitro* studies suggests that *S.* Typhimurium SPI-1 has an impact on invasion. However, studies investigating the role of *S.* Enteritidis SPI-1 *in vivo* are limited. Moreover, research from the murine, bovine and porcine models of salmonellosis indicates that SPI-1 may play a role in breaching the intestinal epithelial layer during the course of an infection. Nevertheless, little is known about the virulence properties of the *S.* Enteritidis SPI-1 T3SS in the colonization of chickens. In addition, recent evidence suggests that *S. enterica* is capable of establishing infection without the presence of SPI-1 [127, 201, 202]. The objective of this study was to investigate the contribution of the *S.* Enteritidis SPI-1 T3SS in invasion of polarized Caco-2 cells, chicken intestinal explants, and in the colonization of chickens over a period of four days post challenge. Our data indicate that *S.* Enteritidis SPI-1 is important for invasion in polarized Caco-2 cells and intestinal epithelial cells *in vitro*. We also show that a ΔSPI-1 mutant strain is not impaired in cecal colonization of 1 week old chickens. However, the deletion of the SPI-1 region causes a delay in systemic infection.

3.2 Materials and Methods

3.2.1 Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 3.1. *S.* Enteritidis Sal 18 was used as the wild type and parent strain for all gene disruptions. *Escherichia coli* K-12 DH5α strain was used as a negative control for invasion assays. Unless otherwise stated, all strains were grown in Luria Bertani Broth (LB) at 37° C and bacterial cultures were agitated in an orbital shaker. SOC medium (2% Tryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5mM KCl, 20mM Mg, 20mM Glucose) was used following transformation of the lambda red PCR products as described below.

Table 3.1 Bacterial Strains used in this study

Bacterial Strain	Description	Reference
Sal 18	*S. enterica* serovar Enteritidis wild type	[131]
LS 21	Sal 18, ΔSPI-1::*cat*	This study
LS 22	Sal 18, Δ*invG*::*cat*	This study
LS 29	Sal 18, *glms*::*tet*	unpublished data
LS 25	Sal 18, *glms*::*cat*	unpublished data
E. coli DH5α	F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	Invitrogen

3.2.2 Construction of mutants

Deletion mutants were made in the entire SPI-1 region (~40 kb) as well as in *invG* (~1.7 kb) using the phage lambda red one-step inactivation method [203]. Briefly, Polymerase Chain Reaction (PCR) primers (Table 3.2) were designed for the flanking regions of SPI-1 ($SPI\text{-}1_F$ and $SPI\text{-}1_R$) and *invG* ($invG_F$ and $invG_R$), based on the *S. enterica* serovar Enteritidis PT4 NCTC 13349 sequence provided by the Welcome Trust Sanger Institute (UK), and the Chloramphenicol resistance gene sequence on a template plasmid (pKD3). PCR products were transformed by electroporation into competent wild type *S.* Enteritidis expressing the lambda red recombinase (pKD46), under the control of an arabinose inducible promoter. Transformants were incubated in SOC [204] for three hours at 37° C in a shaker and plated initially on LB agar plates with 9 μg/ml chloramphenicol then transferred to plates with 34 μg/ml chloramphenicol to select for antibiotic resistant transformants. The ΔSPI-1 and Δ*invG* deletion mutants were confirmed by PCR using a 2720 Thermal Cycler (Applied Biosystems) and sequencing (Plant Biotechnology Institute) using a 3730 XL DNA Analyzer (Applied Biosystems).

3.2.3 Precipitation of SPI-1 secreted proteins

Overnight cultures of *S.* Enteritidis Sal 18 wild type, ΔSPI-1, and Δ*invG* grown in LB containing 0.3M NaCl were subcultured in fresh medium at a 1:50 dilution and grown for 4 hours at 37° C with low aeration until the OD_{600} reached approximately 1.2. The cultures were centrifuged in Eppendorf tubes at 6,000 x g for 10 minutes (all centrifugation steps were at 4° C, unless stated otherwise). The supernatant was filtered into fresh tubes using a 0.2 μm filter, while the sediment fraction was dissolved in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer until it was ready for use. Chilled 100% Trichloroacetic acid (TCA) was added to the filtered supernatant to 20% (v/v) and the mixtures were incubated on ice for 1 hour. The precipitated proteins were centrifuged at 13,000 x g for 30 minutes. The supernatant was discarded and 50 μl of PBS (136 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4), 20 μl 1.5 M Tris pH 8.8 and 1ml chilled (-20° C) acetone was added. The tubes were centrifuged at 13,000 x g for 15 minutes. The supernatant was discarded and the sediment was washed with 300 μl chilled acetone, followed by centrifugation at 13,000 x

Table 3.2 Primers used in this study; F and R refer to forward and reverse primer, respectively, used to amplify lambda red PCR products.

Primer Name	Sequence (5' to 3')
$SPI\text{-}1_F$	GCT GTC GCG TAT GAA GCG ATT GGG TAT TGA TAA AGA CGC GTT AGC GTA AGT GTA GGC TGG AGC TGC TTC
$SPI\text{-}1_R$	ATA TGG TCT TAA TTA TAT CAT GAT GAG TTC AGC CAA CGG TGA TAT GGC CCA TAT GAA TAT CCT CCT TA
$InvG_F$	TCG GCG TTT CGC CGC GGA AAT TAT CAA ATA TTA TTC AAT TGG CAG ACA AGT GTA GGC TTG GAG CTG CTT C
$InvG_R$	GCC GGG GAC AAT ATT CTG GAA AAT GAA ATA CCG GAG GTT GAG CCA GGA ACA TAT GAA TAT CCT CCT TA

g for 10 minutes. The acetone was discarded and the dried pellet was dissolved in 100 µl SDS-PAGE sample buffer and stored until it was ready for use.

3.2.4 Cloning of *sipD* and purification of His-tag SipD

The *sipD* gene was amplified using primers that were designed based on the *S.* Enteritidis PT4 NCTC 13349 sequence provided by the Welcome Trust Sanger Institute (UK). The gene was cloned downstream of a phage T5 promoter into a His-tag pQE-30 vector (Qiagen). SipD was over-expressed in *E. coli* M15 (Qiagen) and purified using a nickel charged resin (Qiagen). Polyclonal anti-sera against SipD were raised in New Zealand white rabbits obtained from Charles River Canada.

3.2.5 Western immunoblots

Proteins secreted under SPI-1 inducing conditions were separated by SDS-PAGE and transferred to a nitrocellulose membrane using a semi-dry transfer apparatus (BioRad) according to the manufacturer's instructions. The membranes were blocked overnight in TBST (8.8 g/L of NaCl, 0.2 g/L of KCl, 3 g/L of Tris base and 500 µL of Tween-20, pH adjusted to 7.4) in 3 % skimmed milk powder at 4° C. The membranes were washed four times with TBST with 10 minute incubations each time. Rabbit polyclonal anti-SipD serum was diluted in TBST (1:5000) and incubated with the membranes for 40 minutes at room temperature. This was followed by four washes with TBST (as described above). Secondary alkaline phosphatase labeled goat anti-rabbit IgG (KPL) was diluted in TBST (1:5000) and incubated with the membranes for 40 minutes at room temperature. The membranes were washed twice with TBST, followed by a wash with AP Buffer (12.11 g/L of Tris base, 5.84 g/L of NaCl, 1.04 g/L of $MgCl_2$ $6H_2O$, pH adjusted to 9.5). To develop the membranes, 33 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma) and 33 µl of nitroblue tetrazolium (NBT) salt (Sigma) were added to 10ml of TBST. This mixture was applied to the membranes and they were allowed to develop at room temperature. The reaction was stopped by rinsing several times with ddH_2O.

3.2.6 Cell culture

Caco-2 cells were grown in Hyclone's DMEM (Fischer) with 10% FBS (Seracare) and 1% non-essential amino acids (Invitrogen), at 37° Celsius and 5% CO_2. To obtain polarized monolayers, Caco-2 cells were seeded onto Transwell inserts (24 mm diameter, 0.4 μm pore size, Corning) for about 21 days. The cells were used for gentamycin protection assays once the trans-epithelial resistance (TER) was determined to be between 250 and 300 ohms cm^{-2} as described elsewhere [194]. All assays were performed in triplicate and were repeated twice.

3.2.7 Invasion assay using polarized Caco-2 cells

Invasion was assessed using the gentamicin protection assay as described elsewhere [205]. Briefly, polarized Caco-2 cells were apically infected with either Sal 18 wild type (LS 29), ΔSPI-1 (LS 21), Δ*invG* (LS 22), or *E. coli* DH5α strains grown in LB at an approximate MOI of 100 for 1 hour. For competition experiments, the cells were apically infected with a 1:1 ratio of wild type and ΔSPI-1 or wild type and Δ*invG* strains (total MOI of 100). The Caco-2 cells were washed three times with 200 μl PBS to remove excess bacteria. The cells were then incubated for 2h with DMEM containing gentamicin (400 μg/ml) to kill extracellular bacteria. The cells were washed two times with 200 μl PBS and lysed with 1% Triton. Serial dilutions were plated on LB Agar containing either tetracycline (5 μg/ml) for the wild type strain or chloramphenicol (34 μg/ml) for the ΔSPI-1 and Δ*invG* strains. For competition experiments, serial dilutions were plated in duplicate on LB Agar containing tetracycline and LB Agar containing chloramphenicol. The invasiveness of the wild type strain (approximately 10^5 cfu/ml) was accepted to be 100%, while the invasiveness of the mutant strains was calculated as a percentage of the wild type using a similar approach as published previously [206].

3.2.8 Measurement of trans-epithelial resistance

The TER was measured using a Millicell – electrical resistance system (Millipore) to determine cell monolayer health and polarity as described elsewhere [194]. For bacterial invasion assays, the TER was measured prior to infection of the polarized Caco-

2 cells with *S.* Enteritidis as well as after gentamicin treatment, to determine the effect of the bacterial strains on the trans-epithelial resistance.

3.2.9 Chicken intestinal tissue explants

Small intestinal and cecal tissue samples were obtained from chickens to assess *S.* Enteritidis invasion as described elsewhere [196]. Briefly, the tissue samples were placed on biopsy disks (25.4 x 2 mm, Fisher) with the mucosal side facing up in 6 well tissue culture plates (Corning) containing DMEM (Sigma) supplemented with 10% FBS. The tissues were co-infected with 10^7 cfu/well of *S.* Enteritidis wild type and ΔSPI-1 strains at a 1:1 ratio for 1 hour to allow for bacterial invasion. The plates were incubated at 41° C with gentle shaking (90 rpm) in an air-tight container under 95% O_2. The samples were washed in DMEM and then incubated with DMEM containing gentamicin (400 μg/ml) for 1 hour, washed in DMEM and homogenized in PBS. Serial dilutions were plated on Brilliant Green Agar (BGA) containing either tetracycline (5 μg/ml) or chloramphenicol (9 μg/ml) to enumerate the bacteria. All assays were performed in triplicate and were repeated at least 3 times. The invasiveness of the wild type strain was accepted to be 100% (approximately 10^5 cfu/ml), as described above, while the invasiveness of the mutant strain was calculated as a percentage of the wild type strain. Samples of the explants in all experiments were subjected to histopathological analysis and the integrity of the intestinal epithelial layers was confirmed.

3.2.10 Passage of strains

S. Enteritidis wild type (LS 25) and ΔSPI-1 (LS 21) strains were passaged through 1 week old specific pathogen free (SPF) chickens prior to the colonization experiments. Isolates were obtained and processed from the liver or spleen as described below and confirmed by PCR and antibiotic selection.

3.2.11 Infection of 1-week old chickens

SPF eggs were obtained from Charles River Laboratories (USA) and were incubated for 21 days until hatch at the Department of Poultry Science (University of Saskatchewan). The chicks were transferred and housed in isolation rooms at VIDO for

the duration of the experiment. The birds were screened using fecal swabs that were plated on Brilliant Green Agar (BGA) to test for the presence of *Salmonella* species. At one week of age birds were randomly divided into two groups containing 40 birds each. The birds were orally challenged with 0.5ml (1 x 10^{10} cfu) of either wild type *S.* Enteritidis or the ΔSPI-1 mutant grown in LB that was administered using an oral gavage needle (18 gauge x 1.5 inches) down the throat. Ten birds from each group were euthanized on days 1, 2, 3 and 4 post challenge. Samples from the liver, spleen and cecal content were weighed, homogenized in saline (0.85 % sodium chloride) and serial dilutions were plated on BGA to determine bacterial counts. In addition, homogenates from the liver and spleen were enriched by incubation in Selenite Broth overnight at 37° C. Enriched samples were streaked on BGA to determine if they contain *Salmonella* species.

3.2.12 Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5.0. The mean percentage of invasion was analyzed using a one-way ANOVA and Bonferroni's Multiple Comparison Test was used to compare the different groups. The recovery of *S.* Enteritidis from the cecal contents, liver and spleen based on direct plating was analyzed by a non-parametric analysis using the Kruskal-Wallis test. Dunn's Multiple Comparison Test was used to compare the different groups. Enrichment data from the liver and spleen was analyzed using the chi-squared test. Fisher's Exact Test was used to compare the different groups. A *P* value less than 0.05 was considered significant.

3.3 Results

3.3.1 The ΔSPI-1 and Δ*invG* strains are impaired in the secretion of SipD

In order to determine if the *S.* Enteritidis mutants constructed were impaired in their ability to secrete SPI-1 T3SS proteins, western blots of bacterial cell lysates and culture supernatants were probed with serum against the His-tag derivative (38.5 kDa) of the SipD protein, the gene of which is encoded by SPI-1 [207]. As expected, SipD (37 kDa) was expressed in the wild type strain and was found in both the supernatant and

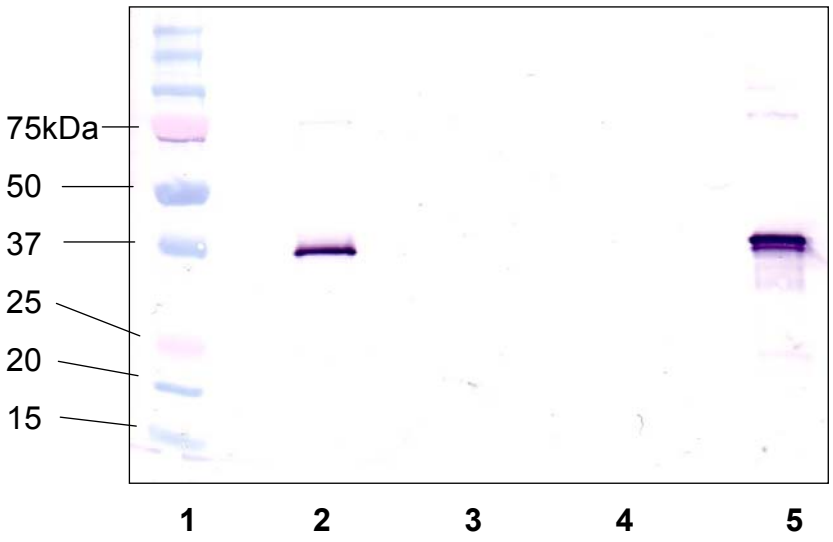
cellular fractions (Figures 3.1A and 3.1B, lane 2). Likewise, SipD was not detected in either the supernatant or cellular fractions of the ΔSPI-1 strain (Figures 3.1A and 3.1B, lane 3). The Δ*invG* strain was unable to secrete SipD into the culture supernatant (Figure 3.1A, lane 4), though the protein was present in the cellular fraction (Figure 3.1B, lane 4).

3.3.2 Salmonella Pathogenicity Island-1 is important for efficient invasion in polarized Caco-2 cells and causes a reduction in the trans-epithelial resistance

S. Enteritidis ΔSPI-1 and Δ*invG* mutant strains were tested for their ability to invade polarized Caco-2 cells relative to the wild type strain, using the gentamicin protection assay. The ΔSPI-1 and Δ*invG* mutant strains were recovered at lower levels relative to the wild type strain, while the *E. coli* strain (control) was unable to invade the Caco-2 cells. Invasion by the ΔSPI-1 and Δ*invG* mutant strains was 6.02% and 8.79%, respectively, of the wild type colony forming units (Figure 3.2A). The TER (Figure 3.2B), on the other hand, was significantly reduced in the wild type infected group (50.0% of the initial value) as compared to the ΔSPI-1 group (82.6% of the initial value), while the TER of the Δ*invG* infected group (70.8% of the initial value) was also reduced, though not to wild type levels, indicating that SPI-1 is important for tight junction disruption. The TER of the control group incubated with *E. coli* was reduced to 67.0% of the initial value.

Using competition studies, polarized Caco-2 cells were infected with a 1:1 ratio of either *S.* Enteritidis wild type and ΔSPI-1 or wild type and Δ*invG* to test the ability of the mutant strains to invade Caco-2 cells in the presence of the wild type strain. ΔSPI-1 and Δ*invG* strains were impaired in invasion as compared to the wild type strain, confirming what was seen in Figure 3.2A (single strain infection). The ΔSPI-1 and Δ*invG* strains showed reduced invasion (6.28% and 5.31%, respectively) relative to the wild type strain colony forming units (Figure 3.3A). The TER (Figure 3.3B) of the co-infected groups (wild type and ΔSPI-1 or wild type and Δ*invG*) was similar (54.0% and 58.0% of the initial TER, respectively) to that of the wild type infected group (50.0% of the initial TER). This was expected since the co-infected groups contained the wild type strain.

A



B

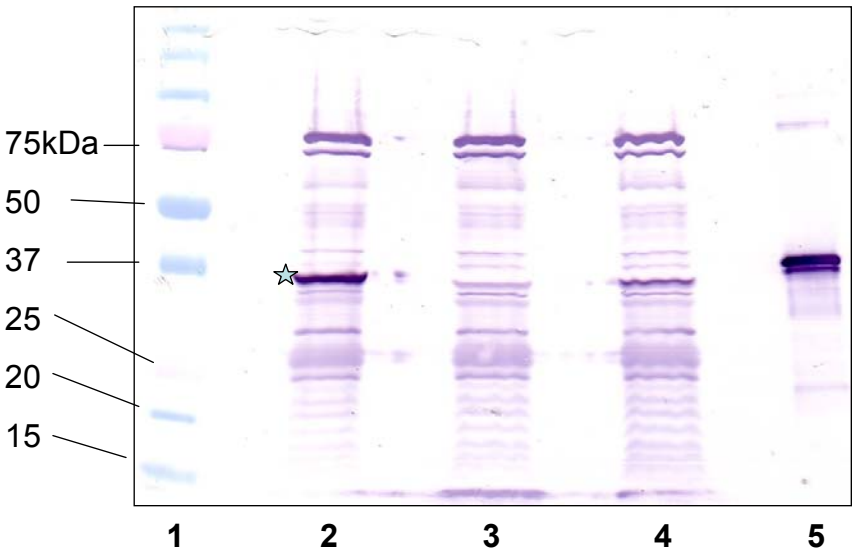


Figure 3.1 Western blot analysis of bacterial culture supernatants (A) and pellet fractions (B) of wild type Sal 18 and the mutant strains constructed, using rabbit anti-His tag-SipD polyclonal serum. Lane 1: prestained protein marker, Lane 2: wild type Sal 18, Lane 3: Sal 18 Δ SPI-1, Lane 4: Sal 18 Δ *invG*, Lane 5: Purified His-tag SipD (38.5 kDa). The SipD protein (37 kDa) was present in the supernatant of the wild type strain (A, lane 2) but not in the Sal 18 Δ SPI-1 and Sal 18 Δ *invG* strains (A, lanes 3 and 4, respectively). In the pellet fractions, SipD was present (*) in the wild type strain pellet (B, lane 2), but not in the Sal 18 Δ SPI-1 pellet (B, lane 3). The Sal 18 Δ *invG* pellet contained some SipD, but not as much as the wild type (B, lane 4).

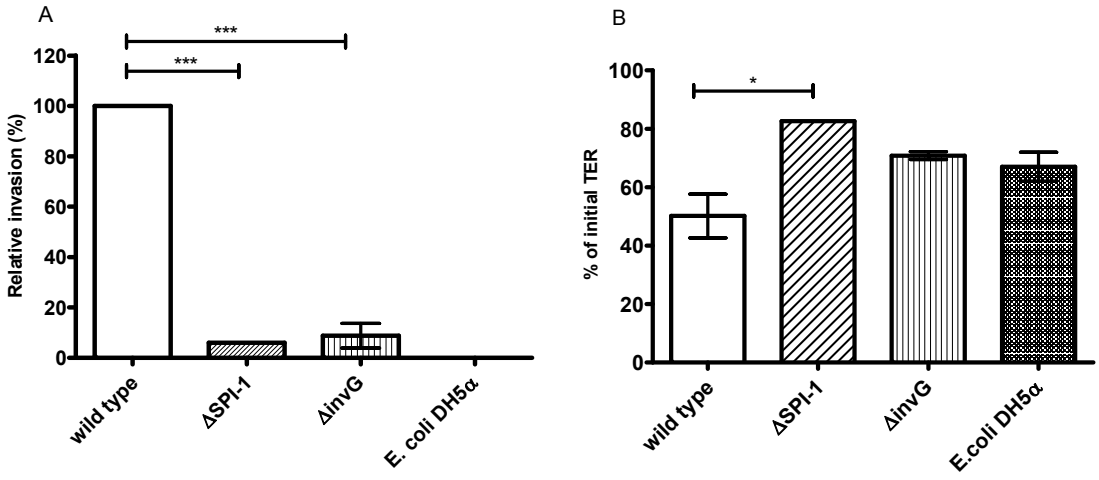


Figure 3.2 Single infection of polarized Caco-2 monolayers by either *S.* Enteritidis wild type, ΔSPI-1, Δ*invG* or *E. coli* DH5α at an moi of 100. Values represent means ± the standard error of the mean of at least two independent assays performed with triplicate wells. (A) Invasion is expressed as a percentage of the wild type strain (B) Change in TER is expressed as a percentage of the initial TER value. ***, $P < 0.001$.

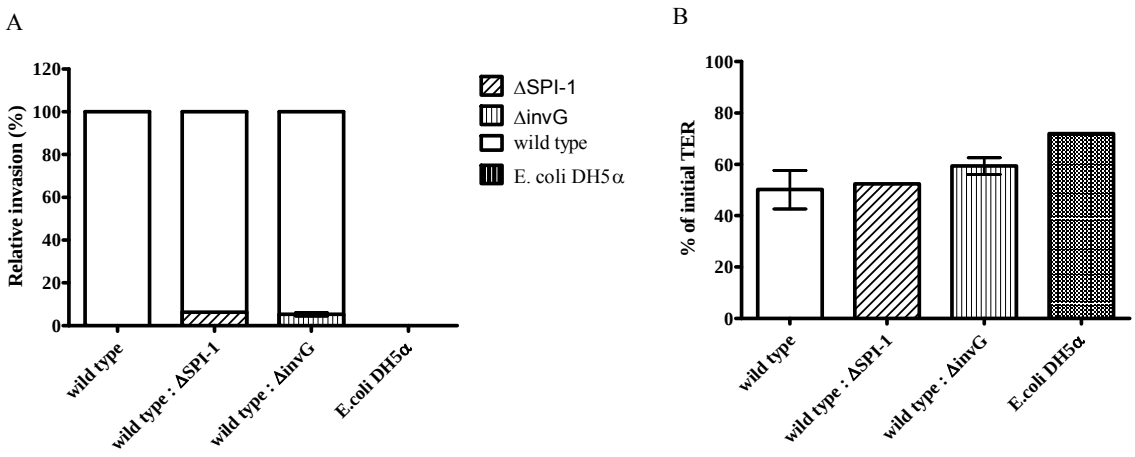


Figure 3.3 Mixed infection of polarized Caco-2 monolayers by *S.* Enteritidis wild type and ΔSPI-1 or wild type and Δ*invG* at a 1:1 ratio with an moi of 100. Values represent means $\pm$ the standard error of the mean of at least two independent assays performed with triplicate wells. (A) Invasion is expressed as a percentage of the wild type strain (B) Change in TER is expressed as a percentage of the initial TER value.

3.3.3 The SPI-1 deficient strain is less invasive relative to the wild type strain in chicken intestinal tissue explants

Chicken intestinal tissue samples were used to test invasion of the *S*. Enteritidis wild type and ΔSPI-1 strains using the gentamicin protection assay as described above. In this competition experiment, the SPI-1 deficient strain was recovered at a lower rate from both the cecal and small intestinal tissue explants (29.3% and 17.2%, respectively) as compared to the wild type strain (Figure 3.4), while the *E. coli* strain (control) was not recovered (data not shown). This provided more evidence to suggest that SPI-1 plays a role in breaching the chicken intestinal epithelial barrier. However, the invasion defect of the ΔSPI-1 strain was relatively smaller in comparison with the invasion defect observed in polarized Caco-2 cells.

3.3.4 Cecal colonization levels were similar in both the wild type and ΔSPI-1 challenged groups

One week old birds were orally challenged with 1 x 10^{10} cfu of either the wild type strain or the ΔSPI-1 strain as described in materials and methods. Birds were euthanized on days 1, 2, 3 and 4 post challenge. We did not see any statistical difference between the cecal colonization (Figure 3.5) of the wild type and the ΔSPI-1 challenged groups over the duration of the experiment.

3.3.5 The deletion of SPI-1 results in delayed systemic infection in chickens

Analysis of the bacterial load in the liver (Figure 3.6A) revealed that on the first day post-challenge, there was very little detectable *S.* Enteritidis wild type and ΔSPI-1 strains, suggesting that the systemic spread of the bacteria was still in its early stages. This idea is supported by data from enriched samples (Figure 3.6B) which revealed that on day one post challenge, 9/10 livers were infected in the wild type challenged group, while only 3/10 livers ($P < 0.05$) were infected in the ΔSPI-1 challenged group. Two days post challenge the recovery of the ΔSPI-1 strain (Figure 3.6A), as evident from direct plating, was significantly lower ($P < 0.05$) in comparison to the wild type strain

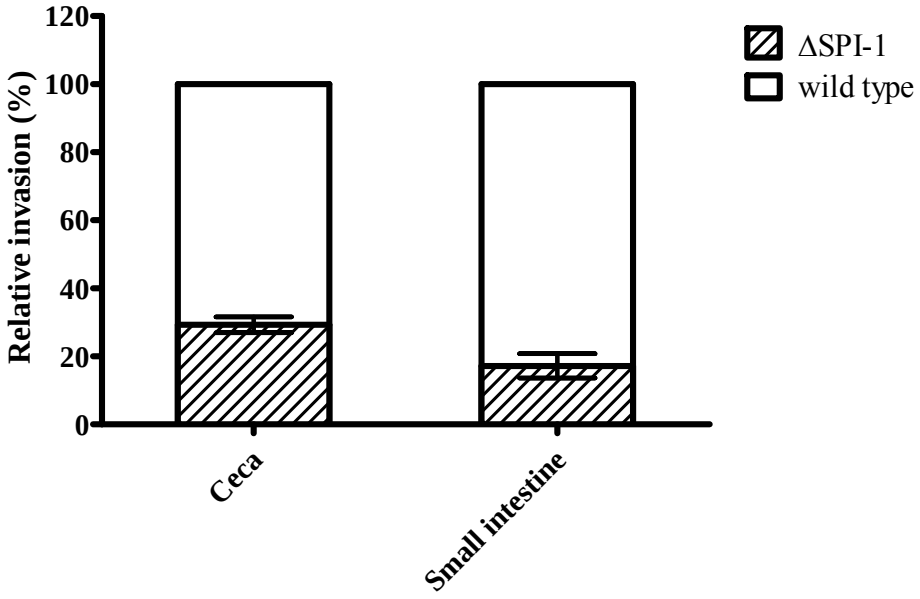


Figure 3.4 Invasion of chicken cecal and small intestinal explants by *S*. Enteritidis wild type and ΔSPI-1 strains at a 1:1 ratio. Values represent means of at least three independent assays ± the standard error of the mean performed with triplicate wells. Invasion is expressed as a percentage of the wild type strain.

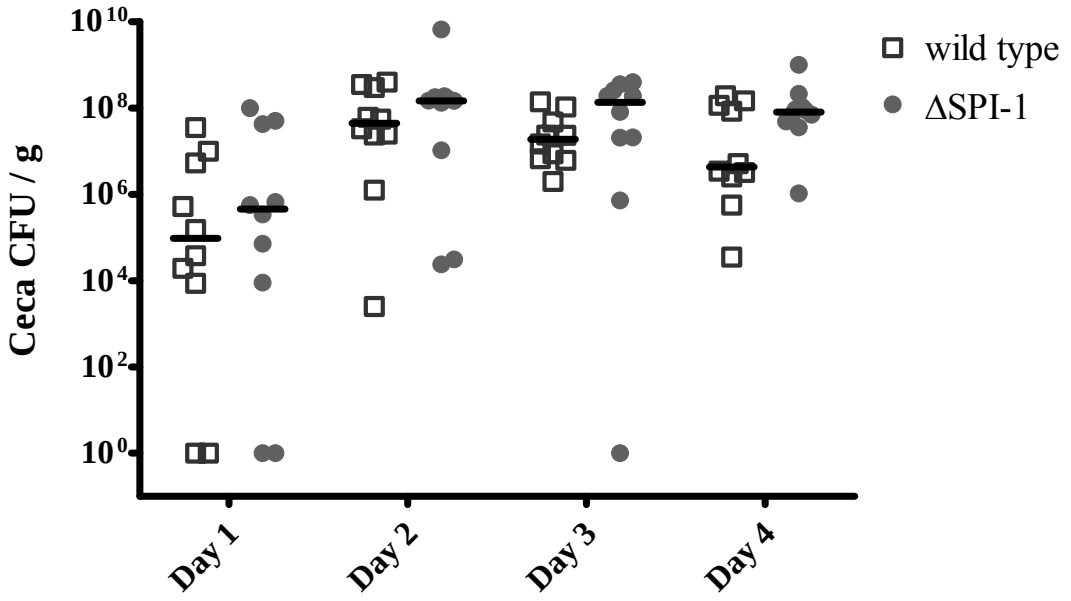


Figure 3.5 Colonization of ceca from 1 week-old chicken by either wild type *S.* Enteritidis or ΔSPI-1. The chickens were orally challenged with 1 x 10^{10} cfu of either wild type or mutant. 10 birds per group were euthanized on days 1, 2, 3 and 4 post-infection.

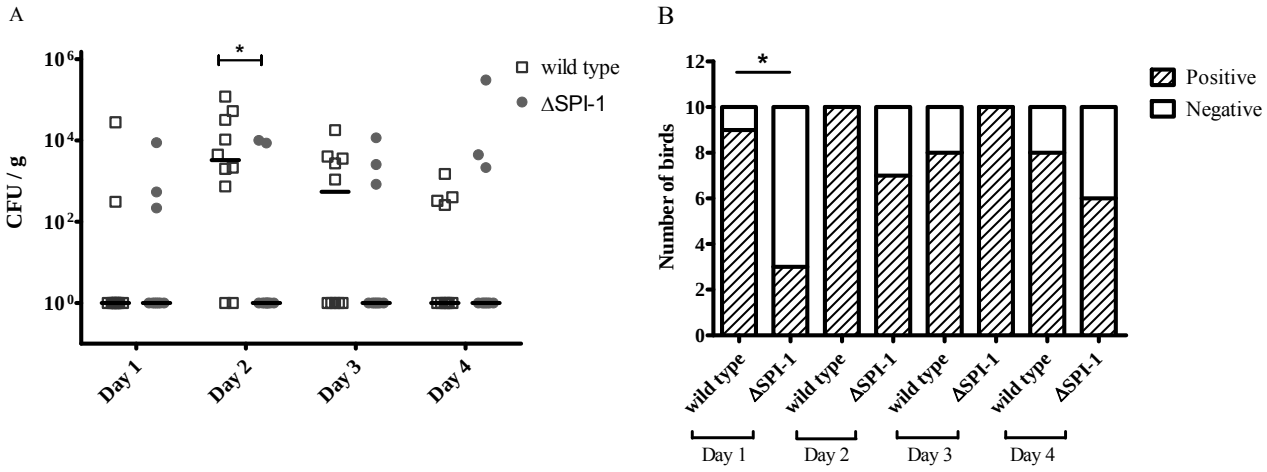


Figure 3.6 Systemic infection of the liver in 1 week old-chicken challenged with 1 x 10^{10} cfu of either wild type *S.* Enteritidis or ΔSPI-1. Ten birds were euthanized per day in each group. (A) viable counts of *Salmonella* from direct plating represented as median cfu/g values (B) number of birds positive for *Salmonella* following enrichment. *, $P < 0.05$.

(3.30×10^3 CFU/g). However, liver enrichment data (Figure 3.6B) demonstrated that the number of livers that were positive in the ΔSPI-1 challenged group (7/10) were only marginally lower than those in the wild type challenged group (10/10). On day 3 post challenge the levels of both the wild type and ΔSPI-1 strains (Figure 3.6A) based on direct counts, followed a similar trend. Data from enriched samples (Figure 3.6B) suggested that the infection was starting to clear in the wild type challenged group on the third day (8/10 livers were positive), while 10/10 livers were positive for *Salmonella* in the ΔSPI-1 inoculated group. After 4 days, the bacterial levels (Figure 3.6A) in both the wild type and ΔSPI-1 challenged groups was mostly below the detection limit using direct plating, which demonstrated that a clearing of the infection was in progress. As for the enriched liver samples (Figure 3.6B), 8/10 livers were still positive for the *Salmonella* wild type strain, compared to 6/10 livers in the ΔSPI-1 challenged group. This suggests that the ΔSPI-1 strain was initially slower in spreading systemically, but also started to clear faster relative to the wild type strain.

The recovery of *S.* Enteritidis wild type and ΔSPI-1 strains from chicken spleens was similar to what was observed in the liver. On the first day post challenge (Figure 3.7A) the infection was still at its early stage and therefore the median group CFU recovery, as determined by direct plating, was below the detection limit in the wild type and ΔSPI-1 challenged birds. Similarly, the data from enriched spleen samples (Figure 3.7B) suggested that there was little difference in the number of spleens that were positive for *Salmonella* in both groups. On day two post challenge, the recovery of bacteria in the wild type challenged group was 7.20×10^2 cfu/g., while that of the ΔSPI-1 challenged group remained unchanged (Figure 3.7A). Likewise, data from enriched samples showed that the number of spleens that were positive for wild type *Salmonella* were slightly higher (7/10) than that of the ΔSPI-1 challenged group (4/10). On the third day after challenge, bacterial levels in the spleen, measured by direct plating (Figure 3.7A) and after enrichment (Figure 3.7B), by both the wild type and the ΔSPI-1 strains followed a similar pattern as on day two. On the last day after challenge (Figure 3.7A), only a few birds were found with countable *Salmonella* on BGA in either the wild type or

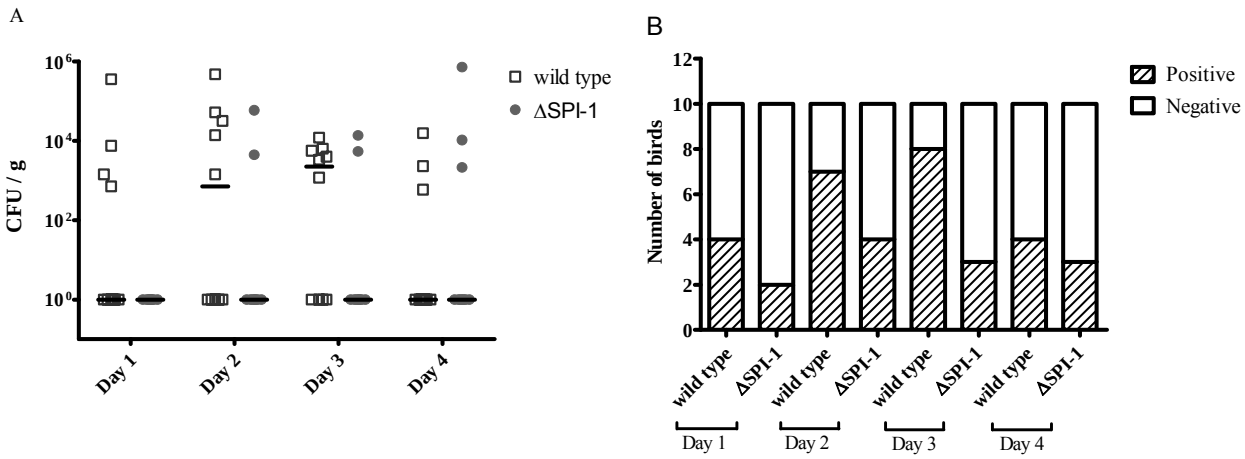


Figure 3.7 Systemic infection of the spleen in 1 week old-chicken challenged with 1 x 10^{10} cfu of either wild type *S.* Enteritidis or ΔSPI-1. Ten birds were euthanized per day in each group. (A) viable counts of *Salmonella* from direct plating represented as median cfu/g values (B) number of birds positive for *Salmonella* following enrichment.

ΔSPI-1 challenged groups. In accordance with this finding, data from enriched samples, showed a marginal difference between the number of spleens that were positive in both wild type and ΔSPI-1 challenged groups suggesting that the infection was clearing. The values described above represent median group numbers after statistical processing. For CFU, recovered from individual birds, please see Figures 3.5, 3.6 and 3.7.

3.4 Discussion

The role of SPI-1 in the pathogenesis of *Salmonella enterica* has been studied in detail in both the mouse enterocolitis and the bovine enteritis models of infection [86, 127, 128, 201, 208]. However, the contribution of SPI-1 is still not fully understood in the chicken colonization model. The goal of this study was to determine the role of *S.* Enteritidis SPI-1 in invasion using polarized Caco-2 cells as well as chicken intestinal explants, and to test the effects of a SPI-1 deletion mutant on cecal colonization and systemic spread of the organism in chickens over a period of 4 days post challenge.

We chose to construct deletion mutants in SPI-1 since it has been well documented that the SPI-1 T3SS is essential for the secretion of effector proteins and plays an important role in infection in different animal models [75, 88, 195, 198]. Thus, it would be expected that deletion of the pathogenicity island in its entire form or the knockout of certain individual genes, would not allow the bacterium to form a fully functional T3SS apparatus nor to secrete any of the SPI-1 related effectors in order to cause intestinal invasion and facilitate bacterial uptake. The ΔSPI-1 and Δ*invG* mutants constructed were tested for their ability to secrete effector proteins in-vitro using western blot analysis, which has been previously used in several studies as a functional assay to determine the effect of SPI-1 deletions on secretion [37, 209, 210]. We chose to monitor the secretion of SipD in the supernatant and pellet fractions of the mutant strains, since SipD is secreted using this system and forms part of the SipB/C/D translocase complex at the tip of the SPI-1 T3SS apparatus [75]. The SipD protein was not detected in the supernatant or pellet fractions of the ΔSPI-1 mutant strain, confirming that SipD was not expressed in the cell, since the gene encoding SipD had been deleted. In addition, SipD was not detected in the supernatant fraction of the Δ*invG* strain, as suggested by reports

which show that Δ*invG* strains are unable to secrete effector proteins [211]. The presence of SipD in the cellular fraction of the Δ*invG* strain is also expected since the *sipD* gene was not deleted in this strain. Interestingly, the level of SipD present in the cellular fraction of the Δ*invG* mutant strain was much lower than the amount present in the wild type fraction. We speculate that this may be due to the fact that the regulation of SPI-1 involves several regulators, including the main regulator, HilA (encoded on SPI-1). The HilA protein is known to bind directly to promoters on the *inv/spa* and *prg/org* operons on SPI-1, thus activating the expression of several genes encoding components of the SPI-1 T3SS. Activation of the *inv/spa* operon by HilA eventually leads to the activation of the sic/sip operon, which encodes SipD. Further, activation of the *inv/spa* operon results in the expression of InvF (a positive transcriptional activator) which, along with the chaperone SicA, also induces expression of SPI-1 secreted proteins on the *sic/sip* operon and those located outside SPI-1 [34]. Therefore, it is possible that the deletion of *invG* affects the expression of genes in the *inv/spa* operon, which ultimately affects downstream genes like *sipD* in the *sic/sip* operon.

In order to examine the contribution of *S.* Enteritidis SPI-1 to invasion, we used polarized Caco-2 cells. This cell line was chosen due to the absence of well characterized chicken epithelial cell lines. Moreover, Caco-2 cells are capable of polarizing on transwells, resulting in well defined apical and basolateral compartments, making them a good model for studying host cell invasion. Caco-2 cells also form well defined brush borders and express several markers that are present on small intestinal villus cells [190]. Further, it has been demonstrated that polarized epithelial cells reveal additional information, with respect to invasion, which would not have been possible with nonpolarized cells [196]. The choice of this cell line was also relevant to this study since *S.* Enteritidis is also a potential human pathogen [160]. Our results from the invasion assays using Caco-2 cells implied that ΔSPI-1 and Δ*invG* mutant strains were impaired in invasion relative to the wild type strain in both the single infection experiments and competition experiments. This was in line with other reports which demonstrated that SPI-1 was important for invasion in cell culture [197, 212-214]. The difference in invasion between the wild type and SPI-1 mutant strains was about 10-fold based on our

invasion assays, which is comparable to what has been observed by others in similar experiments with *S.* Typhimurium and *S. enterica* subspecies *enterica* serovar Typhi [195, 196, 206, 210, 215, 216]. This data also suggested that the role of *S.* Enteritidis SPI-1 in tissue culture models was similar to that observed for *S.* Typhimurium. To our knowledge, this is the first time *S.* Enteritidis ΔSPI-1 and Δ*invG* mutants have been tested (both single and mixed infections) using polarized Caco-2 cells.

The disruption of tight junctions is a strategy used by different bacterial pathogens, including *Salmonella*, to cause virulence, which in turn, damages the epithelial cell structure [217]. We used the TER as an indicator of cell monolayer health, which has been widely used by others to assess the affect of bacterial pathogens on tight junctions [190, 218, 219]. Our results from the invasion assays (single infection) suggest that both *S.* Enteritidis ΔSPI-1 and Δ*invG* mutant strains caused a smaller reduction of TER, in comparison to the wild type strain. This was expected since the SPI-1 mutants used in our study were impaired in the secretion of SPI-1 effector proteins. On the other hand, there was no difference in TER between the wild type alone and co-infected groups, which contained the wild-type and either ΔSPI-1 or Δ*invG* strains. This can be explained by the fact that the co-infected groups all contained the wild type strain, which is capable of secreting SPI-1 effector proteins. The *E. coli* strain also caused a reduction in the TER, which was similar to that caused by the SPI-1 mutant strains. This finding was not expected because the *E. coli* strain used was a non-pathogenic strain that was not able to invade Caco-2 cells in our assays. A possible reason for this could be that *E. coli* K12 may produce unidentified factors that reduce the TER.

Intestinal tissue explants represent a valid model to study invasion since they bridge the gap between tissue culture based assays and animal experiments. They are commonly used to provide more insights into host-pathogen interactions [196, 220, 221]. Our data from the chicken cecal and small intestinal explants suggest that SPI-1 mediated invasion is important for breaching the chicken intestinal epithelial barrier. This is in agreement with data obtained from bovine intestinal explants that were infected with a SPI-1 mutant of *S.* Typhimurium [196]. However, we observed that the difference in

invasiveness between the wild type and ΔSPI-1 strain was smaller as compared to the difference observed in polarized Caco-2 cells. A similar finding reported in bovine intestinal explants [196], implies that other mechanisms besides SPI-1 may also be involved in the process of invasion. We have shown for the first time that SPI-1 mutants are impaired in invasion of chicken cecal and small intestinal tissue explants.

To extend our observations from the *in vitro* studies using SPI-1 mutants, we tested the effect of the ΔSPI-1 mutant on the colonization and systemic spread of the organism in chickens. Only the ΔSPI-1 mutant was tested in chickens, since the Δ*invG* mutant strain was similar to the ΔSPI-1, in that both strains were impaired in the secretion of SipD and showed reduced invasion in polarized Caco-2 cells. In addition, to get a better understanding of the contribution of SPI-1 in chickens, we examined the effect of a SPI-1 deletion over a period of four days after oral challenge with either wild type *S.* Enteritidis or ΔSPI-1 mutant strains. The extended time frame is crucial since it is possible that the absence of SPI-1 may have a pronounced effect on pathogenesis at a certain stage of infection, while at other stages, the effect may not be obvious.

Our data indicate that the absence of SPI-1 does not significantly affect cecal colonization on all four days following oral challenge. This is in agreement with what has been observed in day old chicks orally challenged with a *S.* Typhimurium Δ*spaS* (SPI-1 gene deletion) mutant strain. A similar finding was reported when one-week old chicks were used in the same study. However, cecal colonization levels of the Δ*spaS* challenged group were lower relative to the wild type strain on day 14 post challenge [89]. Likewise, lower levels of cecal colonization were observed in day old chicks challenged with *S.* Enteritidis Δ*hilA* relative to the wild type only after day 6 post challenge [88]. As well, reduced levels of *S.* Typhimurium ΔSPI-1 were reported in cecal tissue of one-week old chickens over a 14 day period [130]. Hence, it is likely that differences between cecal colonization levels of wild type and SPI-1 mutant challenged groups are evident when the colonization is monitored over several weeks. We did not extend the chicken experiments past four days following oral inoculation since we have observed that the chickens start to clear the infection.

Our systemic infection data implies that the absence of SPI-1 affects systemic infection, as detected on days 2 and 3 following oral challenge. However, since the detection limit of viable *Salmonella* is 10^2 cfu/g in our sample processing regime (direct plating without enrichment), it is highly likely that many liver and spleen samples from infected birds appear as false negatives. The systemic infection data following enrichment of the liver and spleen samples provide a better insight into the effect of a SPI-1 deletion on systemic spread of *S.* Enteritidis. It is apparent from the enrichment data that the ΔSPI-1 strain causes a delay in infection of the liver ($P < 0.05$) and spleen on the first day post-challenge. Subsequently, the ΔSPI-1 strain was recovered from the liver and spleen at levels that were similar to the wild type strain. This is in line with what has been observed when chicken were challenged with a *S.* Enteritidis Δ*hilA* strain [88], *S.* Typhimurium Δ*spaS* strain [89] or *S.* Typhimurium ΔSPI-1 strain [130]. However, our results from the systemic infection data are in contrast to what has been seen when three week old chicks were challenged with *S.* Enteritidis Δ*sipD* where none of the spleens were infected on day 3 post-challenge [90]. A likely explanation for this might be that the birds were sampled at one time point and were only based on direct plating. Hence, it would be difficult to determine how the *sipD* mutant would have affected colonization over a longer time period, given the fact that our data indicates that there is a delay in systemic infection initially by the ΔSPI-1 mutant strain. As well, our results are not in agreement with the findings from a study in which the *S.* Typhimurium Δ*spaS* strain was not detected on days 1, 7 and 14 post challenge in the livers of 1 week old chicken, relative to the wild type strain based on direct plating [89]. One possible explanation for this difference is that *S.* Typhimurium and *S.* Enteritidis are two different organisms and thus their method of establishing infection may be different in chickens. Alternatively, the Δ*spaS* strain may have been present, but below the detection limit, which is what we observed in our systemic infection data.

The results from our *in vivo* study imply that the SPI-1 virulence determinant does not play a major part in *S.* Enteritidis infection in chicken. A possible hypothesis for this finding could be that *S.* Enteritidis SPI-1 genes are not expressed *in vivo* in chicken. However, data from our lab indicates that sera from chickens challenged with wild type

S. Enteritidis reacts with purified recombinant components of the SPI-1 T3SS (data not shown), suggesting that the SPI-1 T3SS is expressed during infection of chickens. Our systemic infection results (enrichment) illustrate that the absence of SPI-1 results in a delay in infection relative to the wild type strain. This further confirms the concept that the SPI-1 T3SS comes into play at some point during the process of infection in chicken. Recently, SipA, SopA, SopB, SopD and SopE2 (SPI-1 effector proteins) have been detected in the spleens of mice challenged with *S.* Typhimurium [86]. Although the murine model of salmonellosis is different from chicken, this finding not only confirms that SPI-1 is expressed in-vivo, it also provides support for the role of SPI-1 in systemic infection. A recent report has shown that SPI-1 genes are highly expressed at early and late stages of infection in cultured epithelial cells [222]. This finding, despite being from an in-vitro study, is significant since it provides further evidence to suggest that the role of SPI-1 can be extended to the systemic phase of infection and requires further investigation.

The prevailing view has been that SPI-1 is essential for intestinal pathogenesis in different animal models. However, recent evidence from the bovine and murine models of infection suggests that *S.* Typhimurium can cause infection in a SPI-1 independent manner [127, 201, 223]. Further, it has been reported that SPI-1 mutants retain their ability to invade M cells in a murine gut loop model [224], implying that intestinal invasion may involve other factors, besides SPI-1. In addition, it has been shown that human isolates of *S. enterica* subspecies enterica serovar Senftenberg cause intestinal inflammation despite the fact that they are SPI-1 deficient [202]. Taken together, this, along with our data from the chicken colonization study, may suggest that the SPI-1 T3SS is not a critical factor for crossing the intestinal epithelial barrier in some animals.

Although there are differences between *Salmonella* infections that occur naturally under field conditions and those that occur under experimental conditions, the results from our chicken colonization study, as well as those by others provide insights into the mechanisms by which *Salmonella enterica* serovars Enteritidis and Typhimurium colonize chickens. We chose to use a high dose for oral challenge of chickens, unlike

what the birds would encounter in a natural setting, since we observed very low levels of colonization at lower doses (data not shown), making it difficult to interpret the data. In addition, other groups have also used high challenge doses in chicken experiments [88, 89, 130], thus validating our findings.

In summary, we have shown that *S.* Enteritidis ΔSPI-1 and Δ*invG* were unable to secrete effector proteins using SipD as an indicator of SPI-1 effector protein secretion. The mutants showed reduced invasion of both polarized Caco-2 cells and tissue explants obtained from chicken small intestine and ceca. Our data further suggest that the deletion of SPI-1 does not affect cecal colonization in one week old chicken, but causes a milder and delayed systemic infection, as revealed by samples from the liver and spleen. Additional work needs to be done to determine the individual SPI-1 effectors responsible for systemic infection in chicken.

4.0 PROTECTION OF EPITHELIAL CELLS FROM *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS BY ANTIBODIES AGAINST THE SPI-1 TYPE 3 SECRETION SYSTEM

Excerpts from this chapter have been accepted for publication in the Canadian Journal of Microbiology (Desin *et al*, 2010).

4.1 Introduction

Many *Salmonella enterica* strains represent zoonotic pathogens that are associated with enteric food borne illness. The vast majority of human infections caused by *Salmonella* strains are linked to the consumption of contaminated food or water, which normally results in a self-liming gastrointestinal disease. In rare cases, the disease may become severe requiring antibiotic treatment or even hospitalization. *S*. Enteritidis is one of the most frequently isolated serovars with respect to human disease in North America [225].

During the process of infection, *S*. Enteritidis employs several virulence factors including LPS, flagella, fimbriae and Type 3 Secretion Systems (T3SS). The *S*. Enteritidis T3SS have been shown to be important virulence determinants in different animal species (murine, bovine, porcine and chicken models of infection) [127, 130, 195, 208]. One of the T3SS, encoded on Salmonella Pathogenicity Island 1 (SPI-1), is used to inject effector proteins directly into the host cytosol [65]. This system is required for invasion of non-phagocytic cells in the host gastrointestinal tract [26]. The SPI-1 T3SS is composed of several proteins that span both the bacterial inner and outer membranes. Proteins that are exposed on the bacterial surface include the major T3SS component located in the outer membrane (InvG), the protein forming the needle (PrgI) and the translocon components (SipB, SipC and SipD) that upon contact with the host cell form a pore in the host cell membrane. At least 15 effector proteins (many of which are encoded on SPI-1) are known to be secreted into the host cytosol including SipA, SopB, SopE, SopE2 and SptP. This results in massive host cytoskeletal rearrangements triggering the uptake of the bacterium [28, 61].

Invasion of the intestinal epithelial layer is a crucial step in *S*. Enteritidis pathogenesis, as this allows the bacterium to spread systemically to other sites.

Antibodies against different outer membrane components have been used in vitro in an attempt to block the initial interaction between *S*. Enteritidis and the host. In one study, it was revealed that sera against *S*. Enteritidis LPS but not flagella, prevented entry of the bacterium in Hep-2 cells [226]. The use of polarized Caco-2 and T84 cells demonstrated that anti-sera to both *S*. Enteritidis flagella and formalin inactivated *S*. Enteritidis inhibited invasion [227]. Similarly, antibodies against *S*. Typhimurium LPS have blocked *S*. Typhimurium invasion in MDCK cells [216, 228]. These studies present valuable information that can be used to devise strategies to prevent *S*. Enteritidis infection in animals. However, to date, there is limited data available on the use of antibodies directed against the SPI-1 T3SS as a method of blocking *S*. Enteritidis entry into epithelial cells in vitro. Consequently, the objectives of this study were to examine the role of sera against SPI-1 T3SS proteins in invasion of polarized Caco-2 cells and to identify which components, if any, are major players.

4.2 Materials and Methods

4.2.1 Bacterial strains and growth conditions

S. Enteritidis Sal 18 wild type *att*Tn7::*tet*, *S*. Enteritidis Sal 18 ΔSPI-1::*cat* [188], S. Enteritidis Sal 18 Δ*sipD*::*cat* (constructed using the phage lambda red system) and *S*. Typhimurium (*S*. Typhimurium) SL1344 [194] were used for the invasion inhibition assays. *S*. Enteritidis Sal 18 wild type and the Δ*sipD* mutant strain were used for preparing the lysates for the western blots. All bacterial strains were grown in Luria Bertani (LB) broth at 37° C and were agitated in an orbital shaker.

4.2.2 Preparation of SPI-1 secreted proteins

An overnight culture of the *S*. Enteritidis wild type strain was subcultured in LB containing 0.3 M NaCl at a 1:50 dilution and grown at 37° C with low aeration for 4 hours (O.D. 600 = 1.2). The culture was centrifuged at 6,000 x g for 10 minutes (centrifugation was at 4° C, unless otherwise stated) after which the fraction containing the supernatant was precipitated using chilled 100 % Trichloroacetic acid (TCA) as described previously [229].

4.2.3 Cloning and purification of His-tag proteins

The *invG*, *prgI*, *sipA*, *sipC*, *sipD sopB, sopE and sopE2* genes were amplified under standard PCR conditions and cloned downstream of either (i) a phage T5 promoter into a His-tag pQE-30 vector (Qiagen) or (ii) a phage T7 promoter into a His-tag pET-15b vector (Novagen). Genes cloned in pQE-30 vectors were over-expressed in *E. coli* M15 (Qiagen) cells, while genes cloned in pET-15b vectors were over-expressed in *E. coli* BL21 (Novagen) cells. Over-expressed His-tag proteins were purified using a nickel charged resin as recommended by the supplier (Qiagen).

4.2.4 Generation of rabbit polyclonal anti-sera

Polyclonal anti-sera to InvG, PrgI, SipA, SipC, SipD, SopB, SopE or SopE2 were raised in New Zealand white rabbits obtained from Charles River Canada. Briefly, rabbits were immunized subcutaneously on days 0 (with 400 μg protein in Freund's complete adjuvant), 21 and 42 (with 200 μg protein in Freund's incomplete adjuvant, respectively). Blood was collected from the rabbits on day 52. Serum antibody titers were determined using enzyme linked immunosorbent assay (ELISA).

4.2.5 Cell culture

Caco-2 cells were grown in Hyclone's DMEM (Fischer) supplemented with 10 % FBS (Seracare) and 1 % non-essential amino acids (Invitrogen) at 37° Celsius and 5 % CO_2 in a humidified incubator. The cells were seeded into Transwell inserts (24 mm diameter, 0.4 μm pore size, Corning) and cultured for about 21 days to obtain polarized monolayers. Once the transepithelial resistance (TER) was established to be between 250 and 300 ohms cm^{-2} as described elsewhere [188, 194] the cells were used for invasion inhibition assays. All experiments were performed in triplicate and were repeated at least two times.

4.2.6 Invasion inhibition assay

Invasion was assessed using the gentamicin protection assay as described previously [188]. Briefly, polarized Caco-2 cells were apically infected with the appropriate bacterial strain grown in LB at an approximate multiplicity of infection

(MOI) of 100 for 1 hour, either in the presence or absence of sera (heat inactivated by incubation at 50° C for 30 minutes) against SPI-1 structural proteins at a 1:20 dilution which has been previously used by others [230-232]. Excess bacteria were removed by washing the cells three times with 200 µl PBS. The cells were incubated for 2h with DMEM containing gentamicin (400 µg ml^{-1}) to kill the extracellular bacteria. The cells were washed two times with 200 µl PBS and lysed with 1% Triton. Serial dilutions were plated on LB agar and bacterial colony counts were measured. The invasiveness of the wild type strain without sera (approximately 10^5 cfu ml^{-1}) was accepted to be 100%, while the invasiveness of other groups incubated with sera was calculated as a percentage of the wild type using an approach similar to that previously published [188, 206].

4.2.7 Trans-epithelial resistance

A Millicell – electrical resistance system (Millipore) was used to measure the TER as an indication of the polarity of the Caco-2 cells as described elsewhere [188, 194]. The TER was measured prior to infection of the polarized Caco-2 cells with bacteria as well as after gentamicin treatment, to assess the effect of the bacteria on the transepithelial resistance.

4.2.8 Western blots

Whole cell lysates of *S*. Enteritidis wild type and Δ*sipD* were separated by SDS-PAGE and transferred using a semidry transfer apparatus to nitrocellulose membranes (Bio-Rad) as per the manufacturer's instructions. The membranes were blocked overnight in TBST (8.8 g/L of NaCl, 0.2 g/L of KCl, 3 g/L of Tris base and 500 µL of Tween-20, pH adjusted to 7.4) in 3 % skimmed milk powder at 4° C. The membranes were washed four times with TBST with 10 minute incubations each time. Rabbit polyclonal pre-immune, anti-SipD or SipD depleted sera were diluted in TBST (1:5000) and incubated with the membranes for 40 minutes at room temperature. This was followed by four washes with TBST (as described above). Secondary alkaline phosphatase labeled goat anti-rabbit IgG (KPL) was diluted in TBST (1:5000) and incubated with the membranes for 40 minutes at room temperature. The membranes were washed twice with TBST, followed by a wash with AP Buffer (12.11 g/L of Tris base, 5.84 g/L of NaCl, 1.04 g/L of

$MgCl_2$ $6H_2O$, pH adjusted to 9.5). To develop the membranes, 33 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma) and 33 µl of nitroblue tetrazolium (NBT) salt (Sigma) were added to 10 ml of TBST. This mixture was applied to the membranes and they were allowed to develop at room temperature. The reaction was stopped by rinsing several times with ddH_2O.

4.2.9 ELISA

ELISA plates (Immulon® 2 HB) were coated with 100 ng of antigen per well in coating buffer (12.5 mM Na_2CO_3 and 37.5 mM $NaHCO_3$) for overnight incubation at 4° C. The plates were washed eight times (8x) with ddH_2O and blocked with 200 µl of TBST for 2 hours at room temperature. After washing the plates with ddH_2O (8x), 100 µl per well of rabbit polyclonal sera (1:100) was added to the first row and this was serially diluted along the plate. The primary sera was allowed to incubate for 1 hour at room temperature and washed with ddH_2O (8x). This was followed by the addition of 100 µl of alkaline phosphatase labeled secondary goat anti-rabbit IgG (KPL) at a 1:5000 dilution which was allowed to incubate for 1 hour at room temperature. The plates were washed with ddH_2O (8x) 100 µl of PNPP substrate (Sigma) was added to each well. After incubation at room temperature, the absorbances were read in a plate reader at a wavelength of 405 nm (490 nm as the reference).

4.2.10 Depletion of SipD specific antibodies

To deplete SipD specific antibodies from anti-SipD serum, 1ml of the serum was incubated overnight with 1 mg of purified recombinant SipD protein in an Eppendorf tube with gentle shaking on a nutator. The next day, the serum was stored at -20 C until further use. The depletion of SipD specific antibodies was confirmed by ELISA (section 4.2.9). In addition, SipD depleted serum was tested in an invasion inhibition assay as described above in section 4.2.6.

4.2.11 Statistical analysis

Statistical analysis was performed by using GraphPad Prism 5.0. The mean percentage of invasion from each group in the invasion inhibition assays were analyzed using a one-

way ANOVA. Bonferroni's Multiple Comparison Test was used to compare the different groups in each experiment. For antibody titer determinations (ELISA) the cut-off value was considered to be the average of the blank and two standard deviations. Mean IgG titers were analyzed using a one-way ANOVA as described above. A P value of < 0.05 was considered significant.

4.3 Results

4.3.1 Anti-SipD serum inhibits *S*. Enteritidis entry

To determine the effect of antibodies against the SPI-1 related T3SS components on *S*. Enteritidis invasion in Caco-2 cells, sera against secreted effector proteins were tested first. Polyclonal serum against the secreted fraction of SPI-1 proteins significantly inhibited invasion (14.5 %) relative to the control group (no sera) (Figure 4.1), while antibodies against the individual effector proteins, SopB, SopE and SopE2 did not have an inhibitory effect on invasion. In order to identify the fraction(s) from the pool of polyclonal antibodies directed against total SPI-1 secreted proteins that blocked invasion, antibodies against individual structural T3SS proteins were tested. Pooled anti-PrgI and anti-InvG sera did not affect *S*. Enteritidis invasion (Figure 4.2). Interestingly, pooled sera against SipA, SipC and SipD significantly inhibited invasion (16.3 %) relative to the control group (100 %). Similarly, pooled sera against SipA, SipC and SipD also blocked invasion of *S*. Typhimurium (Figure 4.3), implying that a common strategy could be used to block both *S*. Enteritidis and *S*. Typhimurium invasion in vitro. In order to further investigate the effect of pooled sera against SipA, SipC and SipD on *S*. Enteritidis entry, the sera were individually tested to determine if sera against any or all three recombinant proteins inhibited invasion. The presence of anti-SipD serum significantly reduced *S*. Enteritidis entry into Caco-2 cells (3.95 %) relative to the control group (no sera) (100 %), while sera against SipA and SipC had little effect (79.9 % and 94.4 %, respectively, Figure 4.4). To confirm and validate our findings, anti-SipD serum was incubated with a *S*. Enteritidis mutant strain lacking *sipD*, which has already been shown to be less invasive relative to the wild type strain [212, 229]. As expected from the previous study, antibodies against SipD did not further affect invasion of the mutant strain (2.80%)

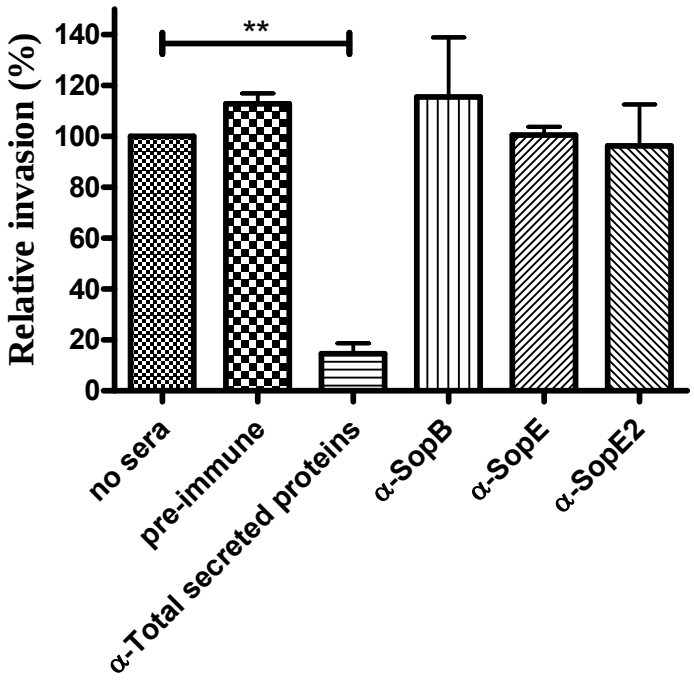


Figure 4.1: Antibodies against total SPI-1 secreted proteins inhibit *S*. Enteritidis invasion. Caco-2 cells were infected with *S*. Enteritidis and were treated with no serum, pre-immune serum, sera against total SPI-1 secreted proteins, and individual components SopB, SopE and SopE2. The efficiency of bacterial entry is expressed as a percentage of the invasion of the control group (no sera). Values represent means $\pm$ the standard error of the mean of at least two independent assays performed with triplicate wells. **, $P < 0.01$.

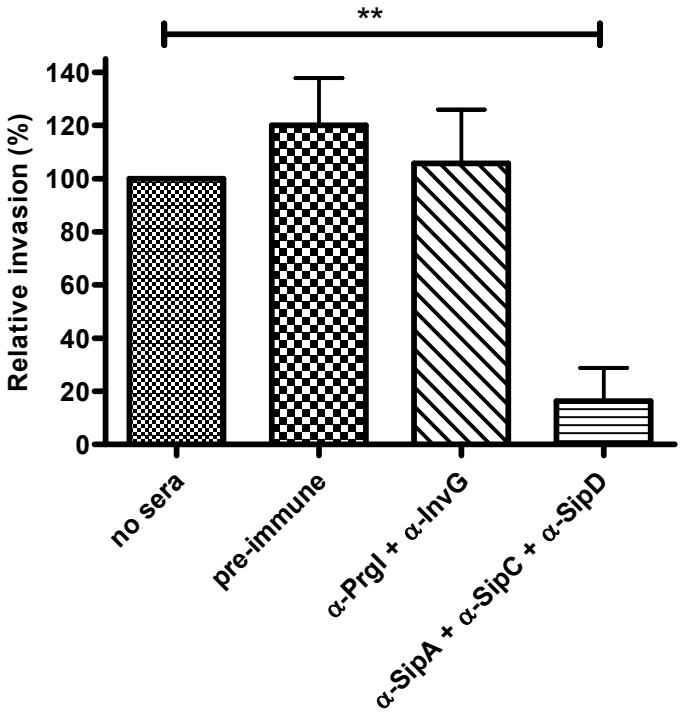


Figure 4.2: Pooled sera against SipA, SipC and SipD inhibit entry of *S*. Enteritidis. Caco-2 cells were infected with *S*. Enteritidis and were treated with no serum, pre-immune serum, pooled sera against PrgI and InvG, or pooled sera against SipA, SipC and SipD. The efficiency of bacterial entry is expressed as a percentage of the invasion of the control group (no serum). Values represent means $\pm$ the standard error of the mean of at least two independent assays performed with triplicate wells. **, $P < 0.01$.

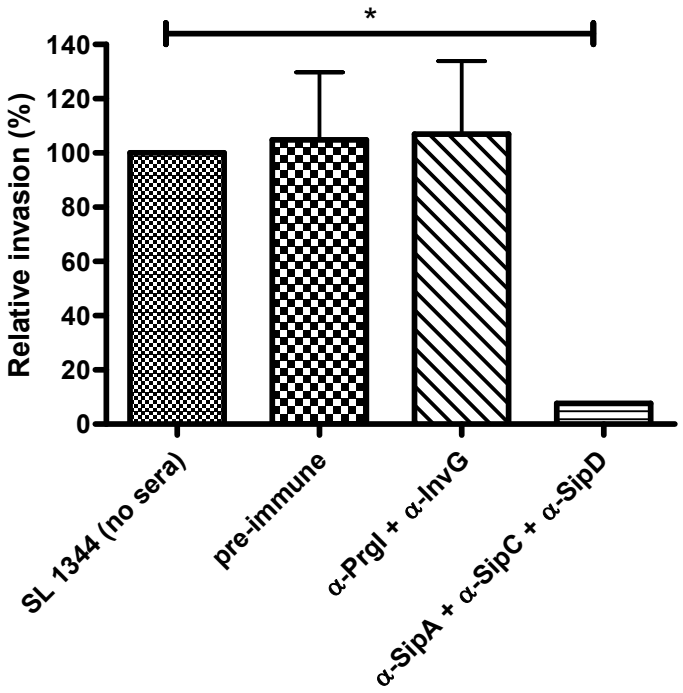


Figure 4.3: *S*. Typhimurium invasion is inhibited by pooled sera against SipA, SipC and SipD. Caco-2 cells were infected with *S*. Typhimurium and were treated with no serum, pre-immune serum, pooled sera against PrgI and InvG, or pooled sera against SipA, SipC and SipD. The efficiency of bacterial entry is expressed as a percentage of the invasion of the control group (no serum). Values represent means $\pm$ the standard error of the mean of at least two independent assays performed with triplicate wells. *, $P < 0.05$.

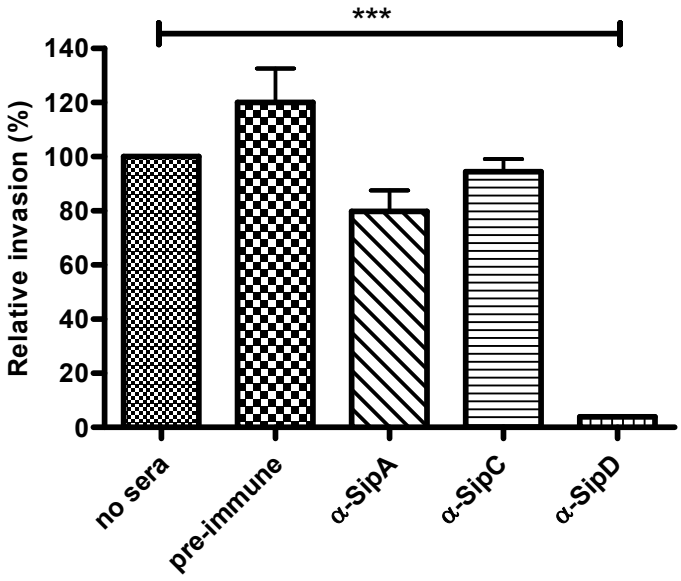


Figure 4.4: Anti-SipD serum inhibits *S*. Enteritidis invasion. Caco-2 cells were infected with *S*. Enteritidis and were treated with no serum, pre-immune serum, and individual sera against SipA, SipC or SipD. The efficiency of bacterial entry is expressed as a percentage of the invasion of the control group (no serum). Values represent means $\pm$ the standard error of the mean of at least two independent assays performed with triplicate wells. ***, $P < 0.001$.

relative to the mutant strain without serum (1.72 % - Figure 4.5). This strongly suggested that the invasion inhibition effect observed by the incubation of anti-SipD serum with *S*. Enteritidis was SipD specific and that any interference with non-SPI-1 related components could be excluded.

4.3.2 Invasion inhibition is SipD specific

In order to demonstrate the specificity of anti-SipD serum, cellular fractions of whole cell lysates from *S*. Enteritidis wild type and a Δ*sipD* mutant strain were probed with pre-immune serum and anti-SipD serum. As expected, pre-immune serum did not detect the SipD protein in the lysates of both wild type and Δ*sipD* mutant strains, nor did it detect the recombinant SipD protein (Figure 4.6A). On the other hand, anti-SipD serum detected the SipD protein in the lysates of wild type *S*. Enteritidis (confirming our previous observation) but not in the corresponding fraction of a Δ*sipD* mutant strain (Figure 4.6B). To further prove the specificity of anti-SipD serum, the serum was depleted of SipD specific antibodies as described in materials and methods. The serum was no longer able to detect the SipD protein in the lysate of wild type *S*. Enteritidis or in the recombinant protein fraction (Figure 4.6C). This was confirmed by ELISA where the depleted serum had significantly lower SipD specific antibody titers relative to the untreated SipD serum (Fig 4.7A). Additionally, the SipD depleted sera was used in an invasion inhibition assay to determine if the invasion inhibition effect observed earlier was SipD specific. As anticipated, the depleted serum restored *S*. Enteritidis entry to 73.3 % relative to the control (no sera) giving further evidence to support the notion that antibodies against the SipD protein block *S*. Enteritidis invasion in polarized Caco-2 cells (Fig 4.7B).

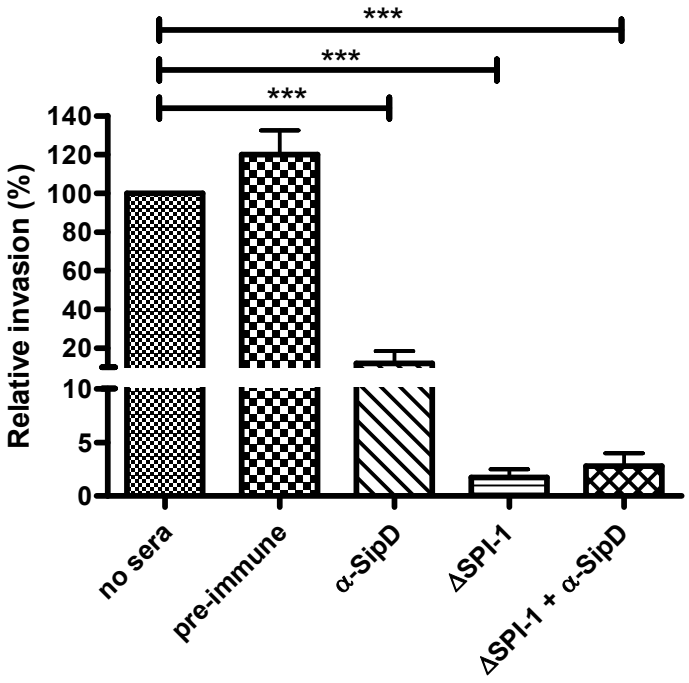


Figure 4.5: Anti-SipD serum mediated inhibition is SPI-1 specific. Caco-2 cells were infected with *S*. Enteritidis (wild type or ΔSPI-1) and were treated with no serum, pre-immune serum, and individual serum against SipD. The efficiency of bacterial entry is expressed as a percentage of the invasion of the control group (no serum). Values represent means $\pm$ the standard error of the mean of at least two independent assays performed with triplicate wells. ***, $P < 0.001$.

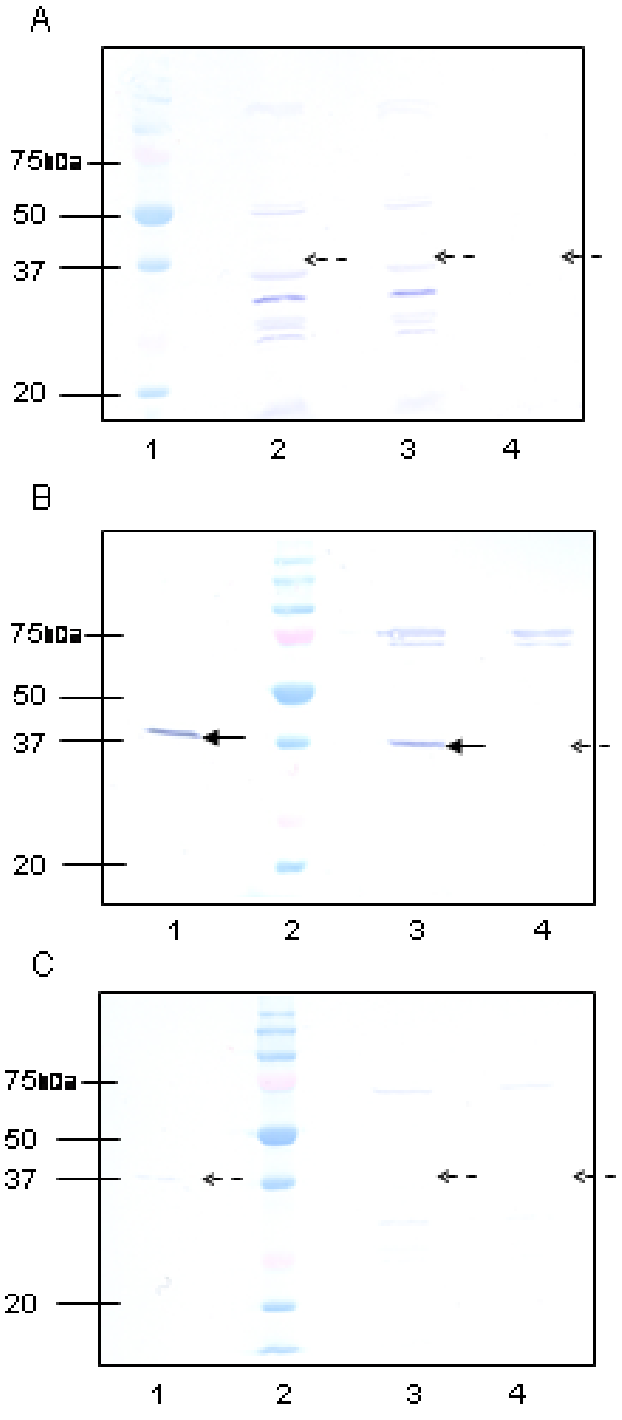

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Figure 4.6: Western blots of the cellular fraction from whole cell lysates of *S.* Enteritidis wild type and the mutant strain Δ*sipD*. (A) rabbit polyclonal pre-immune serum (Lane 1, prestained marker; lane 2, *S*. Enteritidis wild type cell lysate; lane 3, *S*. Enteritidis Δ*sipD* cell lysate; lane 4, purified recombinant His-tag SipD (38.5 kDa)), (B) anti-SipD serum, (C) anti-SipD depleted serum (Lane 1, purified recombinant His-tag SipD (38.5 kDa); lane 2, prestained marker; lane 3, *S*. Enteritidis wild type cell lysate; lane 4, *S*. Enteritidis Δ*sipD* cell lysate. Arrows with solid lines point to positions where the SipD protein has been detected, while arrows with dashed lines point to positions where a band representing SipD would be expected. Pre-immune serum and the SipD depleted serum did not detect SipD (37 kDa) in the whole cell lysate fraction of wild type *S*. Enteritidis (A, lane 2 and C, lane 3) or the recombinant protein (38.5 kDa) (A, lane 4 and C, lane 1). Anti-SipD serum detected SipD in the cellular fractions of wild type *S*. Enteritidis (B, lane 3) but not in the corresponding fraction of the Δ*sipD* mutant strain (B, lane 4).

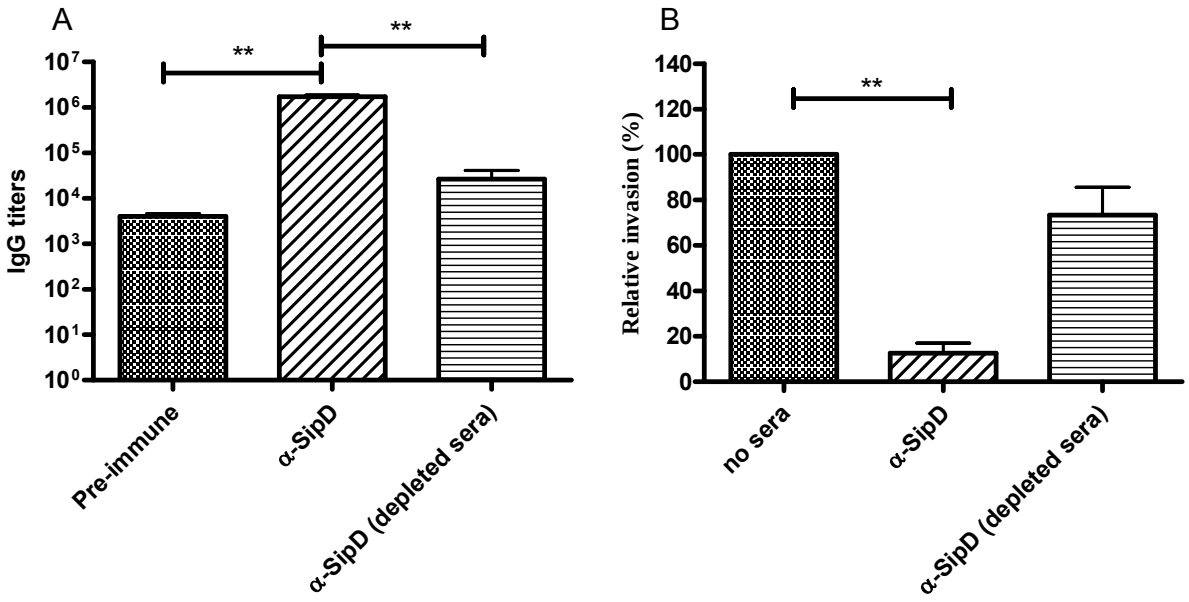

Figure 4.7: Depletion of SipD specific antibodies from anti-SipD serum restores *S.* Enteritidis invasion. (A) Mean IgG titers (means ± the standard error of the mean) of pre-immune serum, anti-SipD serum, and SipD depleted anti-serum. (B) Caco-2 cells were infected with *S.* Enteritidis and were treated with no serum, anti-SipD or SipD depleted anti-serum. The efficiency of bacterial entry is expressed as a percentage of the invasion of the control group (no serum). Values represent means ± the standard error of the mean of at least two independent assays performed with triplicate wells. **, $P < 0.01$.

4.4 Discussion

S. Enteritidis SPI-1 plays a central role as one of the hallmarks in *Salmonella* pathogenesis [25]. This virulence associated system allows the bacterium to gain access to host tissues in systemic sites. At least five effector proteins (SipA, SipC, SopE, SopE2 and SopB) that are secreted by this system are involved in this process, which leads to actin cytoskeletal reorganization, membrane ruffling and bacterial internalization. In addition, the SPI-1 system also contributes significantly to intestinal inflammatory responses. Effector proteins SopB, SopE and SopE2 activate Rho GTPases, which activate other pathways that eventually result in increased production of pro-inflammatory cytokines such as IL-8, leading to an inflammatory response and diarrhoea [61]. Recently, it has been discovered that the SPI-1 system is not only important in intestinal disease, but also in systemic infection [86, 188]. Taken together, this clearly suggests that intervention strategies that interfere with the virulence properties of the SPI-1 secretion system should be an effective tool in controlling *S*. Enteritidis infection.

The aim of the present study was to evaluate the effect of antibodies against selected SPI-1 encoded T3SS proteins on *S*. Enteritidis invasion in polarized Caco-2 cells. The rational behind this approach was the observation by us [188] and others [212, 214] that *S. enterica* SPI-1 mutants have impaired invasion activity in vitro. Polarized Caco-2 cells were used since they are known to establish a well developed microvilli forming brush border that closely resembles the intestinal lumen [190]. This makes the use of this cell line relevant to the present study since the first major barrier encountered by *S*. Enteritidis and other enteric pathogens during host infection is the intestinal epithelial surface. As well, studies have demonstrated that the use of polarized cells reveal additional information with respect to invasion [196] and invasion inhibition [233], which may not be the case with non-polarized cells. For our invasion inhibition assays, initially, we used pooled sera to determine if sera against combinations of different SPI-1 proteins had an effect on *S*. Enteritidis entry. This approach was adopted in order to screen different combinations of sera (effector proteins, translocon proteins and structural proteins). If a particular combination protected Caco-2 cells against *S*. Enteritidis

invasion, we would be able to test sera against individual proteins in that particular group to identify the key player in the process. Moreover, it is unlikely that serum against a particular SPI-1 protein would have an effect when tested individually, but not when tested with a combination of other proteins.

Our data demonstrate for the first time that serum against the SPI-1 SipD protein protects Caco-2 cells against *S*. Enteritidis entry. This is a significant finding since we have identified a mechanism of blocking *S*. Enteritidis invasion by interfering with SPI-1 T3SS mediated virulence in vitro. Our results are also in agreement with recent work that illustrates that the SipD protein is critical for attachment to non-phagocytic cells and is present on the tip of the SPI-1 needle prior to bacterial–host cell contact [38]. The fact that sera against total SPI-1 secreted proteins inhibited invasion was anticipated since we have previously shown, using western blot analysis, that SipD is secreted in the total SPI-1 secreted protein fraction [188]. The absence of an effect on bacterial entry in the presence of sera against SipA, SopB, SopE and SopE2 was expected since the aforementioned proteins are secreted effector proteins whose function is intracellular after being injected into the host cytosol [75]. The observation that serum against SipC, although being part of the SPI-1 translocon, did not affect invasion can be explained by the recent finding which indicates that SipD is expressed prior to bacterial contact, while SipC is expressed only upon contact [38]. Hence, SipD might be easily accessible to antibodies relative to SipC. Moreover, it is possible that SipD plays a more pronounced role in attachment to epithelial cells compared to the latter. The fact that we did not observe an effect on *S*. Enteritidis entry in the presence of sera against PrgI and InvG was expected since these proteins have not been associated with attachment and bacterial entry.

In order to confirm the specificity of the invasion inhibition effect observed in the presence of anti-SipD serum, we tested the effect of this serum on the invasion of a mutant strain lacking *sipD*. For this purpose, we used a ΔSPI-1 mutant strain since the *sipD* gene is encoded on SPI-1. Moreover, SPI-1 mutant strains are less invasive compared to the wild type strain, but still retain their ability to invade tissue culture cells

[195, 196, 206, 210, 215, 216]. As expected, the invasiveness of the mutant strain was not affected by the presence of anti-SipD serum. This suggested that the invasion inhibition effect observed was indeed SipD specific. Moreover, the SipD protein, as well as the other seven SPI-1 proteins used in this study were over-expressed and purified using the same procedure from an *E. coli* K-12 strain that does not have a T3SS. Therefore, it is highly unlikely that antibodies against a protein that co-purified with the SipD protein would cause inhibition of *S*. Enteritidis invasion. Further, the notion that antibodies against SipD affect *S*. Enteritidis entry in Caco-2 cells, is also supported by evidence from studies involving the analysis of T3SS tip proteins (BipD, IpaD and LcrV) in other bacterial pathogens which provide compelling evidence to suggest that the SipD protein is localized to the *S*. Enteritidis needle tip [234-236].

To further investigate the specificity of the antibodies against SipD, we performed western blots using pre-immune and anti-SipD serum. Our results clearly indicate that pre-immune serum does not detect the SipD protein in the lysate from wild type *S*. Enteritidis or the recombinant protein, while SipD anti-serum detected the protein in the aforementioned fractions. This finding implies that the antibodies raised against SipD were highly specific to the protein. Furthermore, SipD specific antibodies were depleted from SipD anti-serum to enable us to investigate the invasion inhibition properties of the depleted serum. As expected, the depleted serum was unable to detect the SipD protein in the lysates of wild type *S*. Enteritidis or in the SipD recombinant protein fraction. However, SipD specific antibody titers were still present in this serum, but at significantly lower levels compared to the untreated serum. This is possible since it is difficult to completely deplete all the SipD specific antibodies. Likewise, when the depleted serum was used in an *S*. Enteritidis invasion inhibition assay, the rate of invasion was restored to approximately 73.3 % of the control group. The fact that the invasion rate did not return to 100 % can be explained by the low levels of SipD specific titers still present in the depleted serum fraction. Taken together, these experiments establish beyond reasonable doubt that the protection of epithelial cells from *S*. Enteritidis entry is mediated by SipD specific antibodies.

Tight junctions form important intercellular structures found in polarized epithelial cells [193]. Many bacterial pathogens, including *Salmonella*, disrupt these junctions to damage the integrity of the epithelial cell layer [217]. We used the TER as an indicator of cell monolayer health since it has been extensively used by others to evaluate the effect of bacterial pathogens on tight junctions [218, 219]. Our results from the invasion inhibition assays illustrate that incubation of anti-SipD with *S*. Enteritidis not only had a significant effect on bacterial entry, but also caused a lower reduction in TER relative to the group with no sera (data not shown). However, it is important to note that invasion does not always result in a reduction in the TER. This observation can be explained by the fact that bacterial invasion takes place very quickly, while the disruption of tight junctions are more noticeable after longer periods of infection [194]. The fact that sera against SipD would cause a lower reduction in TER relative to the control group (data not shown) is expected since SPI-1 effector proteins have been identified as major players in the disruption of tight junctions [190, 194].

In the present study, we used polyclonal IgG antibodies obtained from rabbits immunized with different SPI-1 T3SS proteins. Although, we were able to protect Caco-2 cells from *S*. Enteritidis invasion, an IgA antibody response may be more desirable to prevent invasion at mucosal surfaces since this antibody isotype is resistant to degradation in the harsh protease-rich environment found in mucosal secretions [237]. As well, the levels of IgA antibodies in the intestinal environment are much higher than IgG antibodies, making the former antibody isotype a better candidate at blocking and interfering with *S*. Enteritidis interactions with host cells. Despite the advantages of having an IgA antibody response at mucosal surfaces, we cannot rule out the effect of IgG antibodies in preventing *S*. Enteritidis infection to systemic sites, since recent work suggests that SPI-1 may also contribute to systemic spread of this pathogen [86, 130, 188]. In addition, to date, there is little evidence to suggest that IgA antibodies are required for the clearance of *S*. Enteritidis infection in different animal models [170].

The observation that sera against SipD blocks bacterial entry of *S*. Typhimurium in Caco-2 cells is not surprising, but is significant because both *S*. Typhimurium and *S*.

Enteritidis have a significant impact on human health and are most commonly associated with human infections. This finding is important in designing strategies to control infection by the two aforementioned serovars, since many intervention strategies that are commercially available do not confer protection against both serovars [170]. Consequently, our strategy of blocking invasion via antibodies against SipD does not have to be limited to these two serovars and may be extended to other host-specific and non-host specific *S. enterica* serovars.

In summary, this work has illustrated for the first time that anti-SipD antibodies inhibit *S*. Enteritidis entry in polarized Caco-2 cells, while sera against other SPI-1 T3SS proteins do not. In addition, our results establish that the aforementioned invasion inhibition effect is SipD specific. This data is significant since it adds more support to recent work which indicates that the SPI-1 T3SS is involved in intimate attachment and forms the tip of the needle [38]. As well, from a therapeutic point of view, the SipD protein may be used for protection assays using in vivo models in future experiments.

5.0 IMMUNIZATION OF CHICKENS WITH *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS PATHOGENICITY ISLAND-1 STRUCTURAL PROTEINS INDUCES STRONG HUMORAL RESPONSES AND CONFERS PROTECTION FROM SYSTEMIC INFECTION

In preparation for publication.

5.1 Introduction

Infections by non-host adapted strains of *Salmonella enterica* typically cause gastrointestinal disease in animals [136]. Within this species, over 2500 serovars have been identified [5]. *S. enterica* serovar Enteritidis is one of the most frequently isolated serovars with respect to human infections worldwide [9]. This disease results in significant costs associated with health care and patient recovery. Normally, *S*. Enteritidis infection in humans manifests in gastroenteritis leading to diarrohea, abdominal cramps, vomiting and fever. The disease is self-limiting and usually clears within 2 to 7 days. In rare cases, the disease can become systemic (septicemia) requiring antibiotic treatment as well as hospitalization and may lead to the development of Reiter's Syndrome, which is characterized by pain in the joints, irritation in the eyes and painful urination [12, 238].

Infected poultry meat and eggs are most frequently associated with human illness [239-242]. In Europe studies have revealed that approximately 23.7 % of commercial broiler flocks were positive for *Salmonella* species while 20.4 % of layer flocks were positive for either *S.* Enteritidis or *Salmonella enterica* serovar Typhimurium [160]. Similarly, a recent study examined twenty commercial broiler processing plants across the United States and found that *Salmonella* species were detected on 72.0 % of carcasses at the time of slaughter [243]. Chickens are highly susceptible to infection by *S*. Enteritidis in their first few weeks of life. This translates into intestinal colonization and may also lead to systemic infection. Older birds are less likely to get systemic infection, but do get colonized with *Salmonella* in the gastro-intestinal tract. Once chickens are colonized, they continue to shed the bacterium for long periods [131]. This can lead to carcass contamination by *Salmonella* at the time of slaughter, and subsequent transfer to the consumer via poultry products or directly through contaminated eggs, posing a

substantial threat to human health [160]. Consequently, efforts to reduce the prevalence of *S*. Enteritidis in chickens will ultimately reduce the incidence of human infections [241].

Vaccination, along with other intervention strategies, has been used to reduce the prevalence of *S*. Enteritidis in poultry flocks [170, 244, 245]. Three classes of vaccines exist: live attenuated, killed *Salmonella* and subunit vaccines [170]. Live attenuated strains contain mutations or deletions in genes that are important for metabolism, virulence or survival. They confer several advantages including the possibility of oral delivery, rapid protection, induction of both humoral and cell-mediated responses, and they can be administered to young chicks [170, 246, 247]. A major drawback of live attenuated strains is that they consist of a living organism that is being introduced into the field and it is possible that attenuated *Salmonella* strains may persist in chickens for prolonged periods resulting in their transmission to humans. Hence, there is a school of thought which considers killed *Salmonella* and sub-unit vaccines to be more favourable from a consumer safety perspective. Several groups have demonstrated that killed vaccines induce significant humoral responses and reduce *S*. Enteritidis colonization and egg contamination in chickens [170]. However, the protective antigens in these vaccine formulations are not well defined. Thus, with the continued prevalence of *S*. Enteritidis, there is still a need for improved vaccines that are well defined and capable of inducing significant protection in chickens.

During the process of infection, *S*. Enteritidis uses two specialized nanomachines (T3SS) encoded by SPI-1 and SPI-2, respectively, to inject virulence factors directly into host cells [24]. The SPI-1 secretion system is an important virulence determinant that is mainly associated with breaching the intestinal epithelial surface [25, 26]. The major components of this system that are exposed on the bacterial surface include PrgI (a low-molecular weight protein forming the needle) and the SipB, SipC and SipD proteins (constituting the translocon in the host cell membrane) [24]. Recently, it has been demonstrated that SipD also forms the tip of the SPI-1 T3SS needle and is involved in attachment to epithelial cells in vitro [38]. Therefore, SipD, along with the

aforementioned T3SS structures represent key targets for vaccination. Previously, it was found by other groups and by us that the SPI-1 secretion system is an important virulence factor in chickens [87-89, 130, 188]. In addition, work from our lab has recently demonstrated that antibodies against the tip of the SPI-1 secretion machinery (SipD) block invasion of polarized Caco-2 cells by *S*. Enteritidis (unpublished data). Taken together, this strongly suggests that the SPI-1 secretion apparatus may be an important target for therapeutic measures aimed at inhibiting virulence properties of this system.

The aim of the present study was to evaluate the efficacy of *S*. Enteritidis SPI-1 T3SS proteins in protection against bacterial challenge in layers using an oral challenge model as well as via maternal antibody transfer in progeny derived from vaccinated hens using both an oral challenge and seeder model of infection. We show for the first time that proteins encoded by SPI-1 are immunogenic in chickens and confer significant protection against *S*. Enteritidis colonization of the livers, but not ceca of vaccinates.

5.2 Materials and Methods

5.2.1 Bacterial strains

S. Enteritidis isolate Sal 18 wild type [188] was used for experimental challenge in vaccine trial 1, while *S*. Enteritidis isolate Sal 8 wild type [131] was used for experimental challenge in vaccine trails 2 and 3. The strains were grown in Luria-Bertani (LB) broth at 37° C using an orbital shaker (250 rpm) to an OD_{600} of 0.7.

5.2.2 Protein expression and purification

The *S*. Enteritidis SPI-1 genes, *invG*, *prgI*, *sipC*, *sipD* and *sopB* (encoding structural components and effector proteins) were amplified by PCR (Applied Biosystems) and cloned into either pQE-30 (Qiagen) or pET-15b (Novagen) His-tag expression vectors (unpublished data). The constructs were verified by PCR and sequencing (Plant Biotechnology Institute, Saskatoon). The corresponding proteins were expressed and purified using nickel charged resins (Qiagen) following standard procedures.

5.2.3 Vaccination of chickens

Specific pathogen free (SPF) eggs were obtained from Charles River Laboratories (USA) and incubated at the Department of Poultry Science (University of Saskatchewan). After hatch, the chicks were screened by plating fecal swabs on Brilliant Green Agar (BGA) to ensure that they were *Salmonella* free. Subsequently, the chicks were used for vaccine trial 1.

Vaccine Trial 1

Newly hatched chicks were randomly divided into two groups (30 birds per group) and vaccinated subcutaneously at days 14 and 28 of age with either phosphate buffered saline (PBS) or PrgI + SipD. The concentration of each protein per dose was 50 μg and each vaccine group was formulated with 30 % Emulsigen® D. The birds were orally challenged with 0.5 ml (10^{10} CFU) of *S*. Enteritidis at 35 days of age. Ten birds per group were euthanized on days 1, 2 and 4 after challenge. The liver, spleen and cecal contents were sampled and homogenized in saline (0.85 % sodium chloride). Serial dilutions were plated on BGA to determine the levels of *S*. Enteritidis. In addition, liver and spleen homogenates were incubated in selenite broth overnight at 37°C for enrichment. Enriched samples were plated on BGA to determine the number of samples that were positive for *Salmonella* species. Sera were collected for antibody titer determination at 28 days of age (pre-boost) and after euthanizing the birds (1, 2 and 4 days postchallenge). The experimental design is summarized in Figure 5.1.

5.2.4 Immunization of laying hens

Leghorn chickens were obtained and housed at the Department of Poultry Science (University of Saskatchewan). The hens were screened using fecal swabs by plating on BGA to ensure that they were *Salmonella* free. Subsequently, the hens were used for vaccine trials 2 and 3.

Vaccine Trial 2 – seeder challenge model

Laying hens were randomly divided into three groups (12 birds each) and vaccinated subcutaneously with 0.5 ml of either PBS, AviPro® 109 SE4 (positive control

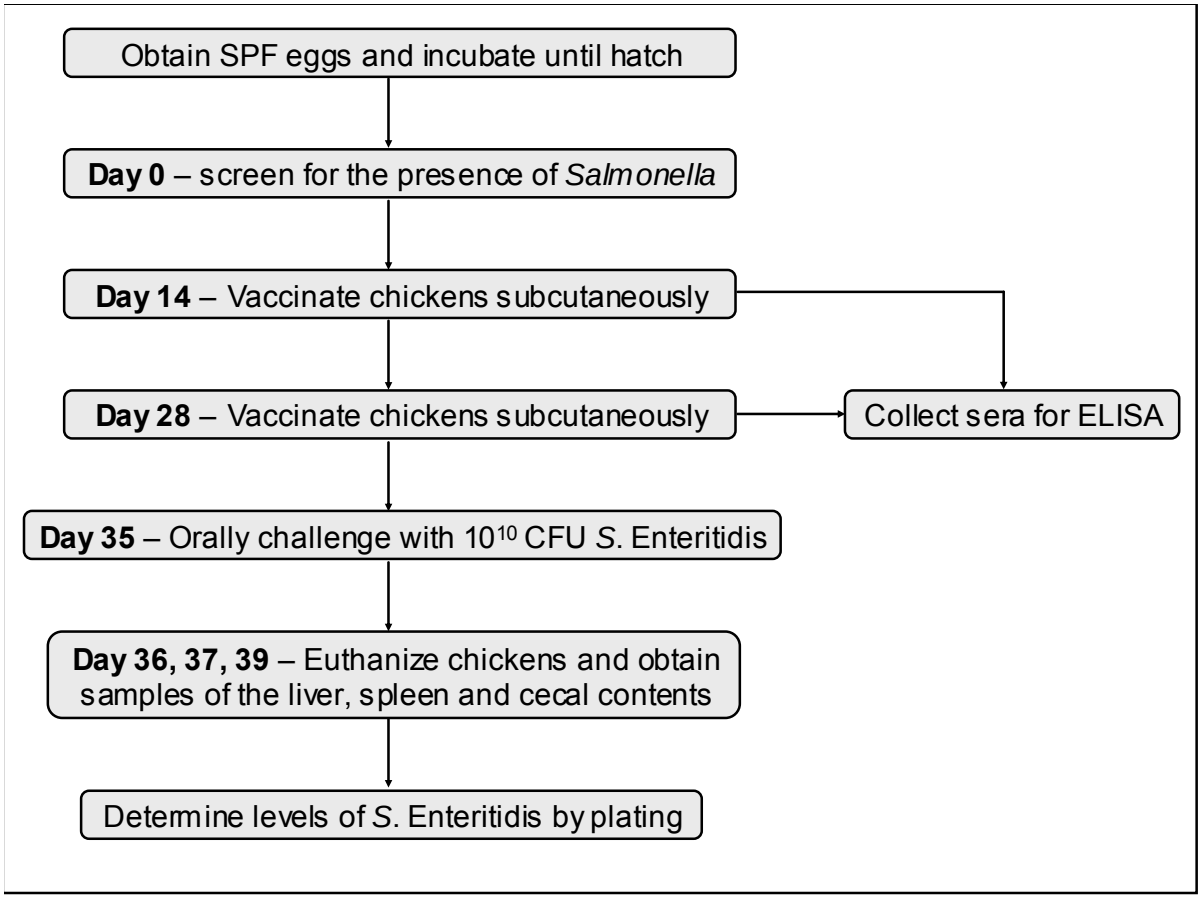

Figure 5.1: Experimental Design for Vaccine Trial 1.

- L.A.H.I.) or PrgI + InvG + SipC + SipD + SopB (20 μg of each protein) on day 0, followed by two boosts on days 21 and 42 (all vaccines were formulated with 30% Emulsigen® D, except AviPro®). Sera were collected from the hens on days 0, 21, 42, and 63 for measuring antibody titers. Subsequently, the hens were artificially inseminated and eggs were collected on days 64, 65 and 71 for determining antibody titers as described below. Three batches of 50 eggs from each group (experiments I, II and III) were set for incubation until hatch at the Department of Poultry Science. For experiments I, II and III, we used a seeder model of infection as described elsewhere [248, 249]. In experiment I, after hatch, the progeny were transferred to isolation rooms at VIDO and approximately 70 % of the chicks per group (seeder birds) were orally challenged with 0.5 ml (10^8 CFU) of *S*. Enteritidis at 1 day of age, while the other chicks were allowed to co-mingle with the rest of the flock (contact birds). Fifty percent of the seeder and contact chicks per group were euthanized on days 2 and 3 postchallenge. Samples from the chicks were collected and processed as described for vaccine trial 1. Similarly, in experiment II, after hatch, approximately 60 % of the chicks per group (seeder birds) were orally challenged with 0.5 ml (10^8 CFU) of *S*. Enteritidis at 1 day of age, while the remaining chicks were allowed to commingle with the rest (contact birds). Fifty percent of the chicks per group (seeder and contact birds) were euthanized on days 2 and 3 postchallenge and samples were collected and processed as described for vaccine trial 1. In experiment III, after hatch, ten percent of the progeny per group (seeder birds) were orally inoculated with 0.5 ml (10^8 CFU) of *S*. Enteritidis at 1 day of age, while the remaining chicks were allowed to commingle with the rest (contact birds). Fifty percent of the chicks per group (seeder and contact birds) were euthanized on days 2 and 3 postchallenge and samples were collected and processed as described for vaccine trial 1. The experimental design is summarized in Figure 5.2.

Vaccine Trial 3 – oral challenge model

Six adult immunized leghorn chickens from each group in vaccine trial 2 were transferred to the VIDO animal care facility and were orally challenged with 0.5 ml (10^8 CFU) of *S*. Enteritidis. The hens were euthanized 2 days postchallenge and samples were collected and processed as described above.

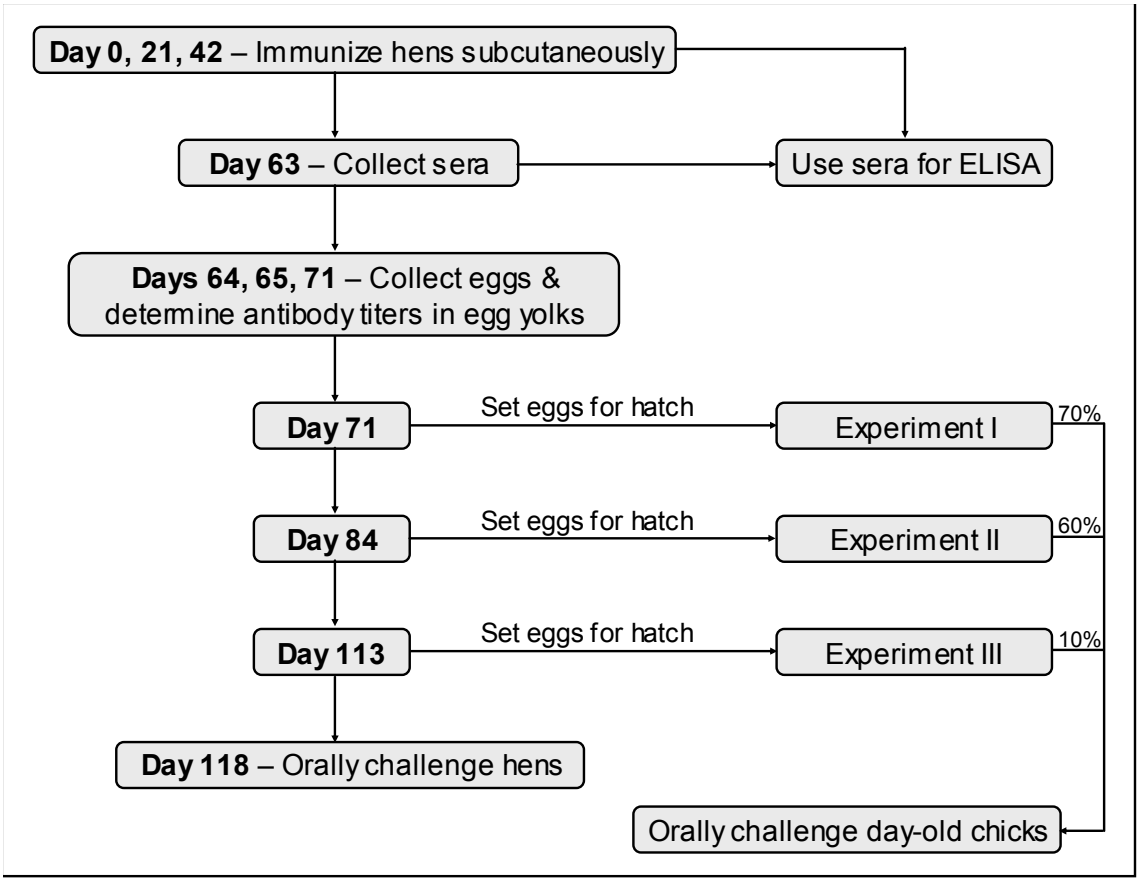

Figure 5.2: Experimental design for Vaccine Trials 2 and 3.

5.2.5 Antibody isolation from chicken egg yolks

One ml of chicken egg yolk was mixed with 4 ml of acidified ddH_2O (pH 2.5) in a 14 ml tube (BD Falcon™). The mixture was incubated on a nutator for 15 minutes at room temperature. The tubes were transferred to a freezer (-20° C) for two days and then allowed to thaw at room temperature. Thereafter, the tubes were centrifuged at 3500 xg for 30 minutes at 4° C to precipitate the lipoproteins. The supernatant was collected and used for antibody titer determination using ELISA.

5.2.6 ELISA

Immulon® 2HB plates were coated with 100 ng of antigen per well in coating buffer (12.5 mM Na_2CO_3 and 37.5 mM $NaHCO_3$) and incubated overnight at 4° C. The plates were washed three times with double distilled water (ddH_2O) and blocked with 100 µl of Borate Buffered Saline - BBS (0.17 M H_3BO_3, 0.12 M NaCl, 0.05 % Tween 20, 1 mM EDTA and 0.25 % BSA) for 30 minutes at room temperature. The plates were washed three times with ddH_2O and chicken sera was added to the first set of wells (1:33). This was serially diluted for 4 fold dilutions down the plate. The sera were incubated for 30 minutes at room temperature after which the plates were washed three times with ddH_2O. The plates were blocked with 50 µl of BBS for 10 minutes at room temperature and washed with ddH_2O three times. Thereafter, 50 µl of horse radish peroxidase labeled goat anti-chicken IgG (KPL) at a concentration of 1:100 was added to each well and the plates were incubated for 30 minutes at room temperature. The plates were washed (3x) with ddH_2O and 75 µl of the ABTS peroxidase substrate (KPL) was added to each well and incubated for 30 minutes at room temperature. The reaction was stopped by adding 75 µl peroxidase stop solution (1:5 in ddH_2O - KPL) per well. The absorbances were read at a wavelength of 405 nm (490 nm as the reference).

5.2.7 Statistical analysis

Statistical analysis was performed by using GraphPad Prism 5.0. A nonparametric analysis using the Kruskal-Wallis test was used to analyze the recovery of *S*. Enteritidis from the cecal contents, livers and spleens based on direct plating. The different groups were compared using Dunn's multiple comparison test. Data from the enrichment of the

liver and spleen samples were analyzed by using the chi-squared test. The Fishers exact test was used to compare the different groups. For antibody titer determinations (ELISA) the cut-off value was considered to be the average of the blank and two standard deviations. Antibody titers were analyzed using a nonparametric analysis (Kruskal-Wallis test) and individual groups were tested using Dunn's multiple comparison test. A *P* value of < 0.05 was considered significant.

5.3 Results

5.3.1 Vaccine Trial 1

Vaccination of chickens with PrgI (major SPI-1 needle component) and SipD (needle-tip) did not affect the levels of *S*. Enteritidis in the cecal contents relative to the control over the duration of the experiment (Figure 5.3). The levels of *S*. Enteritidis in the livers of the vaccinated group were lower on days three and four after challenge relative to the control, with a statistically significant difference ($P < 0.001$) on the fourth day postchallenge based on direct plating (Figure 5.4A). A similar trend was evident in the spleens of the vaccinated group two days postchallenge (Figure 5.4B). On the other hand, data from enriched samples of the livers and spleens revealed that the number of chickens positive for *Salmonella* species was similar in the vaccinated and control groups on all three days post challenge (Figure 5.5). This indicated that vaccination with the SPI-1 proteins, PrgI and SipD, decreased the bacterial load in internal organs, but not the number of birds positive for *Salmonella*. Additionally, immunization with the aforementioned proteins induced significantly higher antibody titers relative to the control group (Figure 5.6).

5.3.2 Vaccine Trial 2

The SPI-1 proteins InvG, PrgI, SipC, SipD and SopB (SPI-1 needle base, needle, translocon component, needle tip and effector protein, respectively) were tested for their ability to protect against *S*. Enteritidis challenge via maternal antibodies using a chicken seeder model. Immunization of hens with the aforementioned proteins, followed by two booster injections, revealed that the SPI-1 proteins induced a significant antibody

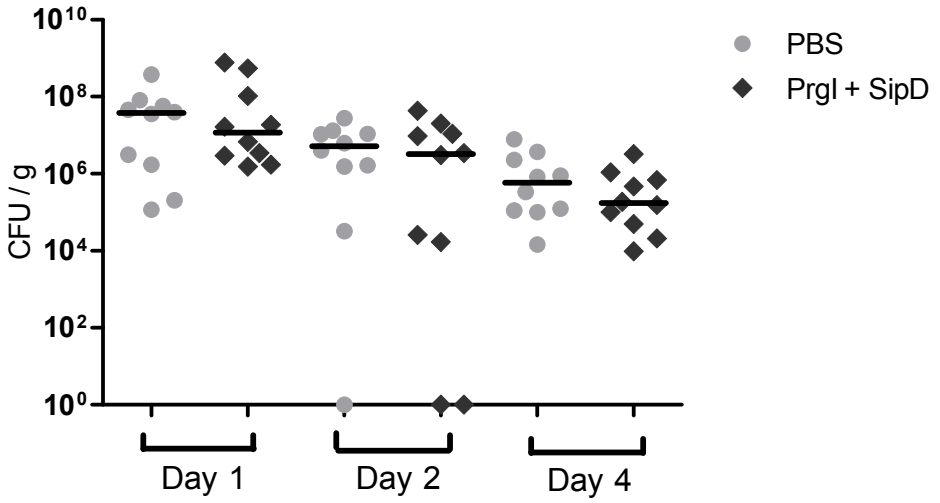

Figure 5.3: Vaccine Trial 1. Levels of *S*. Enteritidis expressed as median colony forming units per gram (CFU / g) in the cecal contents on days 1, 2 and 4 postchallenge (10^{10} CFU) in chickens vaccinated subcutaneously with either PBS or PrgI + SipD.

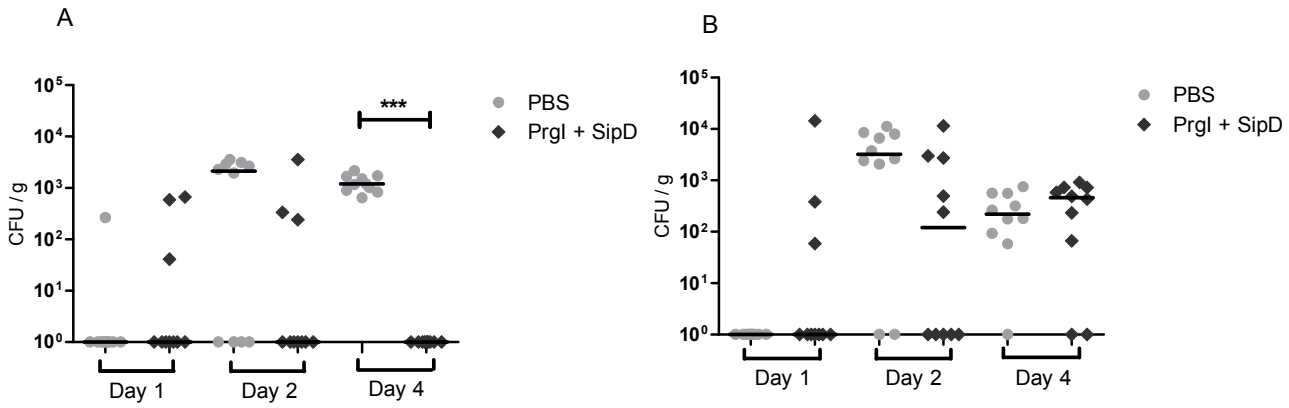

Figure 5.4: Vaccine Trial 1. Levels of *S.* Enteritidis expressed as median colony forming units per gram (CFU / g) in the livers (A) and spleens (B) on days 1, 2 and 4 postchallenge (10^{10} CFU) in chickens vaccinated subcutaneously with either PBS or PrgI + SipD. ***, $P < 0.001$.

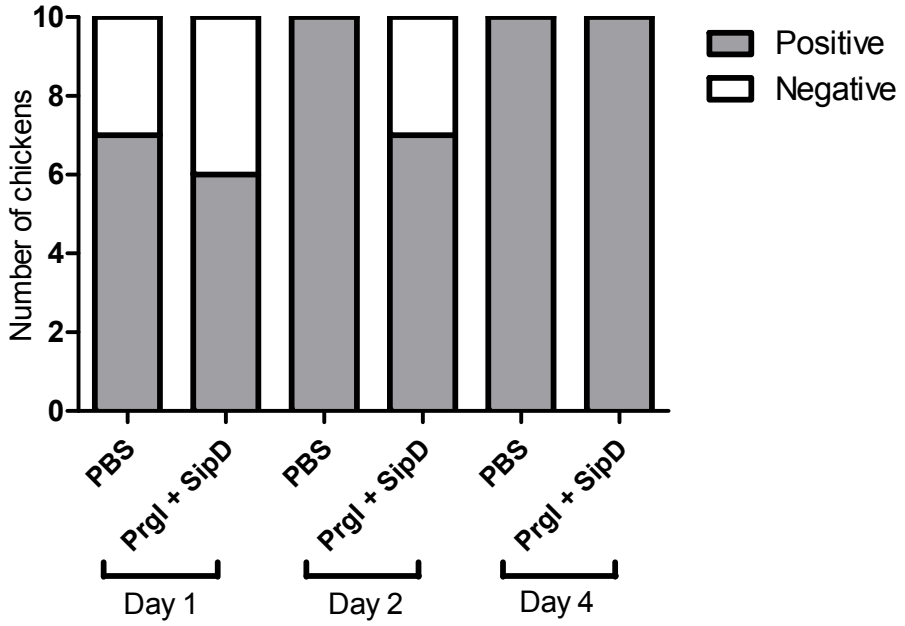

Figure 5.5: Vaccine Trial 1. The number of birds positive for *Salmonella* after enrichment of liver and spleen homogenates (chickens that were positive for *Salmonella* in either liver or spleen were considered as birds positive for *Salmonella*).

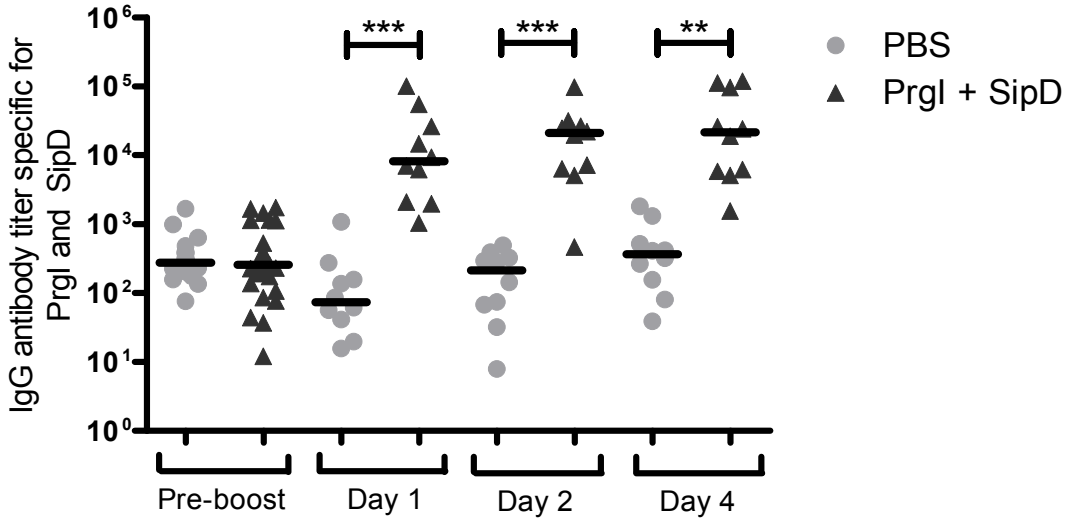

Figure 5.6: Vaccine Trial 1. Median levels of IgG antibody titers specific for PrgI and SipD in sera obtained from chickens vaccinated subcutaneously with either PBS or PrgI + SipD. The chickens were vaccinated twice (14 and 28 days of age) and orally challenged with 10^{10} CFU of *S.* Enteritidis at 35 days of age. **, $P < 0.01$; ***, $P < 0.001$.

response compared to the PBS vaccinated group (Figure 5.7) as in vaccine trial 1. This observation was also true for sera obtained from hens vaccinated with our positive control, AviPro® (Figure 5.8).

In experiment I, 70% of the chicks were orally challenged with *S*. Enteritidis (seeder birds) in each group, while the remaining chicks were allowed to commingle (contact exposed birds). No difference was evident in the levels of *S*. Enteritidis in the cecal contents of both the seeder and contact exposed chicks obtained from the vaccinated group (SPI-1 proteins) relative to the control groups (Figures 5.9A and 5.9B). On day two postchallenge, the median value of *S*. Enteritidis in the livers, based on direct plating, was lower (though not statistically significant) in the progeny (contact exposed birds) that were obtained from the AviPro® (positive control) vaccinates relative to the progeny from the PBS group (Figure 5.10B). In the same way, lower levels of bacteria were observed in the spleens of the progeny from the AviPro® vaccinated group in both seeder and contact exposed chicks compared to the control group over the duration of the experiment (Figures 5.11A and 5.11B). However, immunization of the parent flock with the SPI-1 proteins did not reduce levels of challenge strain in the samples obtained from the progeny (seeder and contact birds).

In experiment II, 60 % of the chicks were orally challenged (seeder birds), while the remaining chicks were allowed to commingle (contact exposed birds). As in experiment I, the levels of *Salmonella* in the cecal contents of the progeny from the SPI-1 vaccinated group were comparable to the levels in the progeny from the AviPro® and PBS vaccinated groups (Figures 5.12A and 5.12B). The recovery of *S*. Enteritidis from the livers and spleens of chicks (contact exposed group) obtained from hens vaccinated with AviPro® was significantly lower on day two postchallenge in the liver and on both days postchallenge in the spleen (Figures 5.13B and 5.14B). This observation did not hold true for the livers and spleens of chicks that were experimentally challenged by oral inoculation (seeder birds) in all the groups (Figures 5.13A and 5.14A). Likewise, vaccination of hens with SPI-1 proteins did not translate into a reduction of the levels of *S*. Enteritidis in the internal organs of their progeny (both seeder and contact exposed birds).

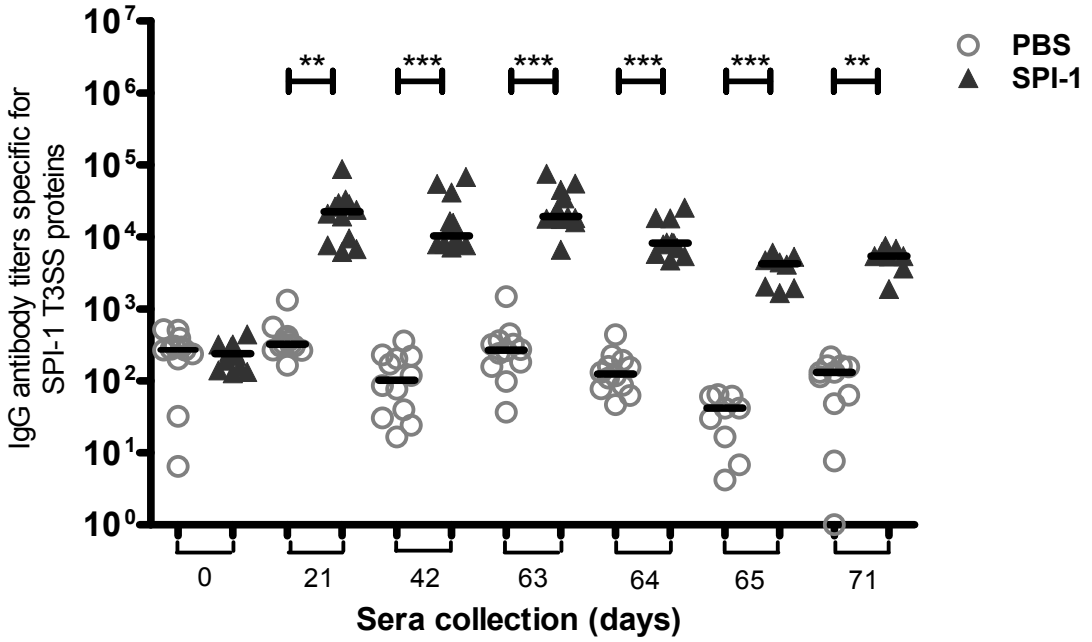

Figure 5.7: Median IgG antibody titers specific for SPI-1 T3SS proteins in sera and egg yolks obtained from laying hens vaccinated subcutaneously on days 0, 21 and 42 with either PBS or SPI-1 T3SS proteins. Sera were collected from the hens on days 0, 21, 42 and 63 for measuring IgG levels. IgG titers were also measured from egg yolks that were extracted from eggs collected on days 64, 65 and 71. **, $P < 0.01$; ***, $P < 0.001$.

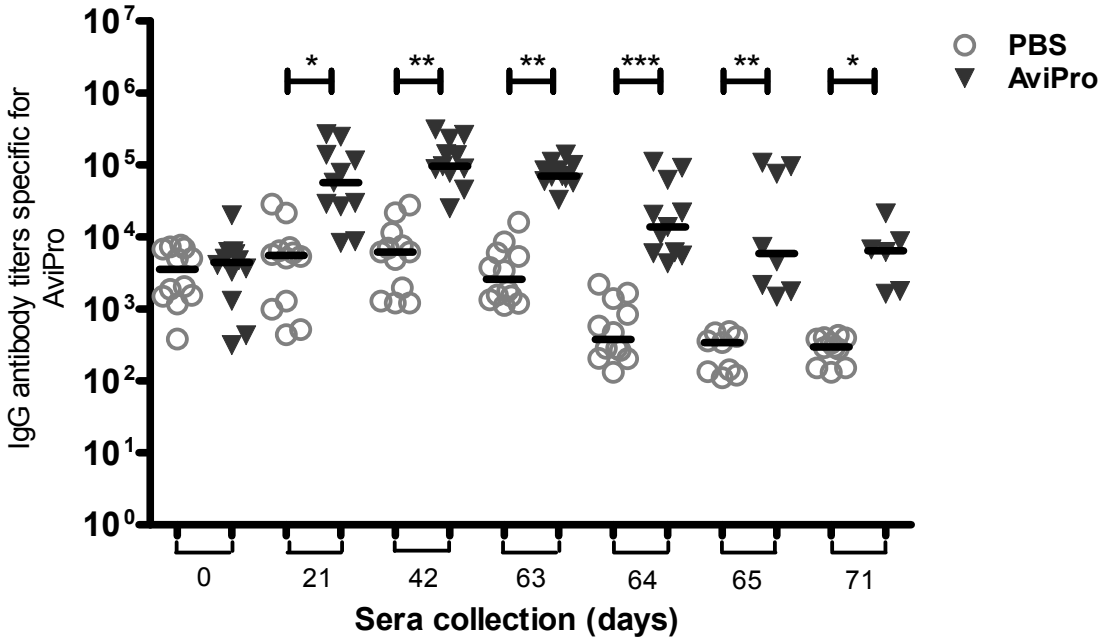

Figure 5.8: Median IgG antibody titers specific for AviPro® in sera and egg yolks obtained from laying hens vaccinated subcutaneously on days 0, 21 and 42 with either PBS or AviPro®. Sera were collected from the hens on days 0, 21, 42 and 63 for measuring IgG levels. IgG titers were also measured from egg yolks that were extracted from eggs collected on days 64, 65 and 71.*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

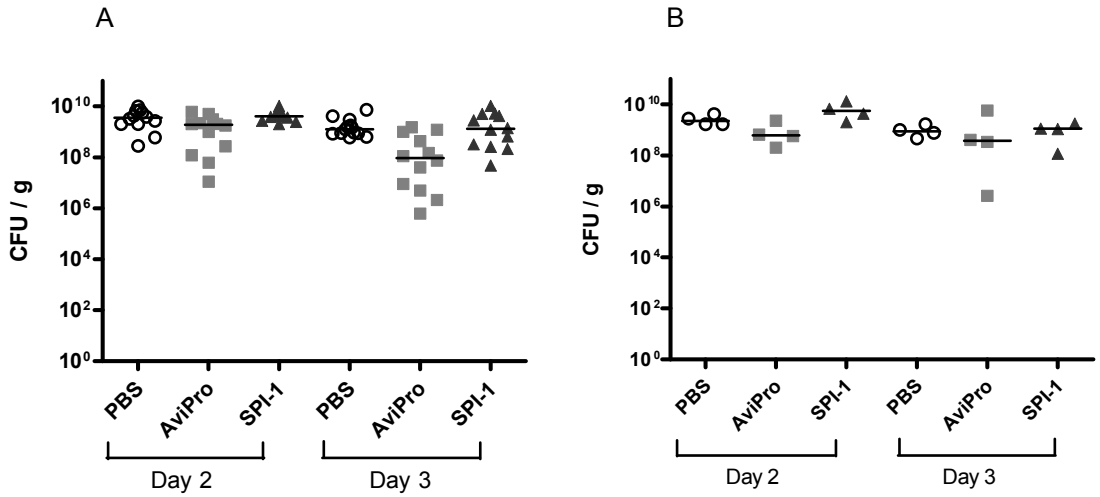

Figure 5.9: Vaccine Trial 2, Experiment I. Levels of *S*. Enteritidis in the cecal contents of progeny obtained from vaccinated hens. Hens were vaccinated subcutaneously with either PBS (open circles), AviPro® (closed squares) or SPI-1 T3SS proteins (closed triangles) where 70 % of the seeder birds were orally inoculated with the challenge strain. Values are expressed as median colony forming units per gram (CFU / g). Bacterial levels in the cecal contents of seeder (A) and contact exposed (B) birds on days 2 and 3 postchallenge (10^8 CFU).

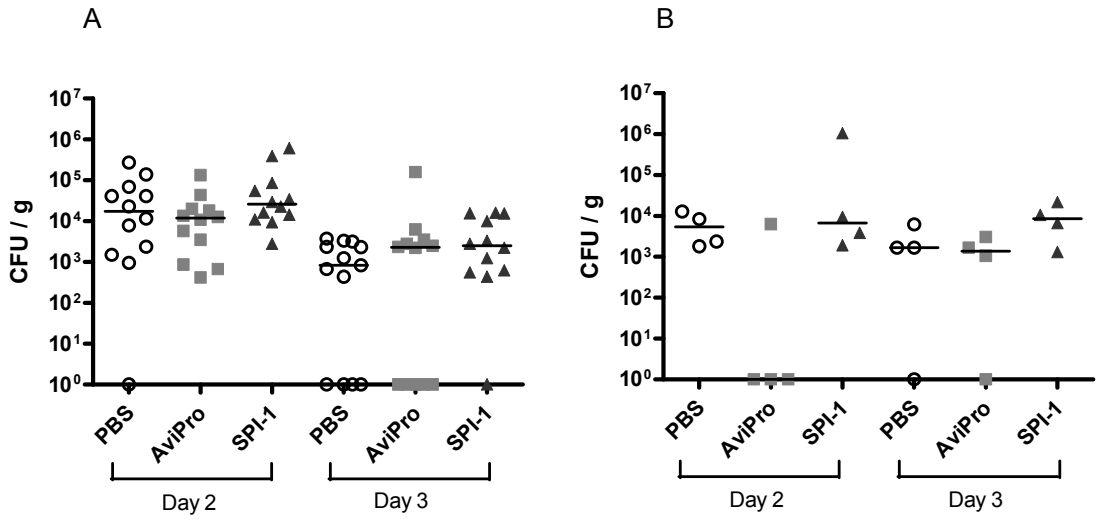

Figure 5.10: Vaccine Trial 2, Experiment I. Levels of *S*. Enteritidis in the livers of progeny obtained from vaccinated hens. Hens were vaccinated subcutaneously with either PBS (open circles), AviPro® (closed squares) or SPI-1 T3SS proteins (closed triangles) where 70 % of the seeder birds were orally inoculated with the challenge strain. Values are expressed as median colony forming units per gram (CFU / g). Bacterial levels in the livers of seeder (A) and contact exposed (B) birds on days 2 and 3 postchallenge (10^8 CFU).

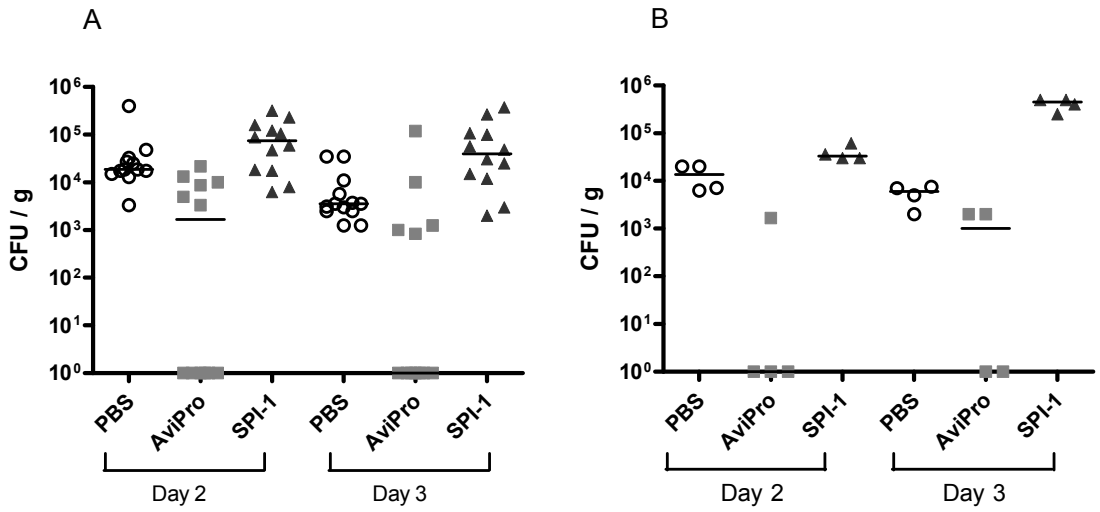

Figure 5.11: Vaccine Trial 2, Experiment I. Levels of *S*. Enteritidis in the spleens of progeny obtained from vaccinated hens. Hens were vaccinated subcutaneously with either PBS (open circles), AviPro® (closed squares) or SPI-1 T3SS proteins (closed triangles) where 70 % of the seeder birds were orally inoculated with the challenge strain. Values are expressed as median colony forming units per gram (CFU / g). Bacterial levels in the spleens of seeder (A) and contact exposed (B) birds on days 2 and 3 postchallenge (10^8 CFU).

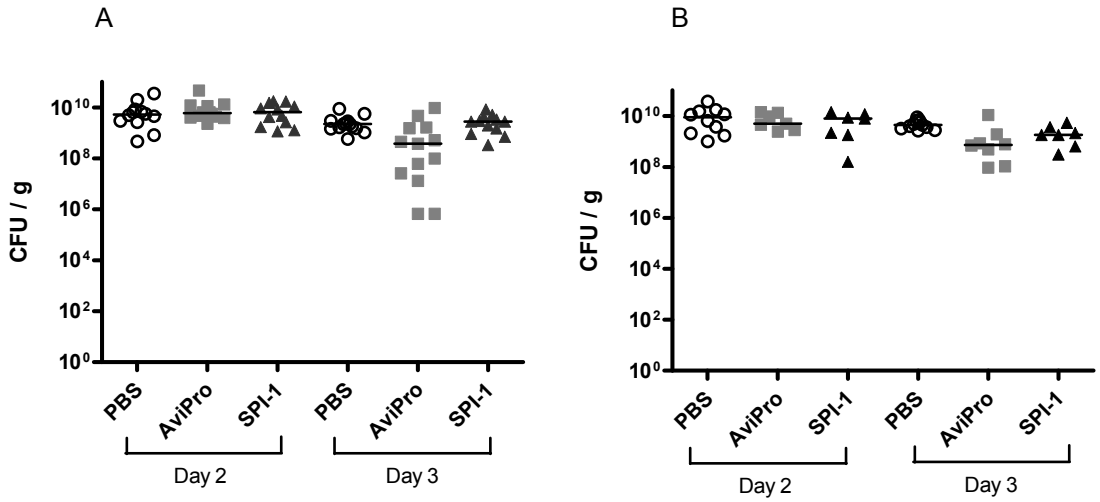

Figure 5.12: Vaccine Trial 2, Experiment II. Levels of *S*. Enteritidis in the cecal contents of progeny obtained from vaccinated hens. Hens were vaccinated subcutaneously with either PBS (open circles), AviPro® (closed squares) or SPI-1 T3SS proteins (closed triangles) where 70 % of the seeder birds were orally inoculated with the challenge strain. Values are expressed as median colony forming units per gram (CFU / g). Bacterial levels in the cecal contents of seeder (A) and contact exposed (B) birds on days 2 and 3 postchallenge (10^8 CFU).

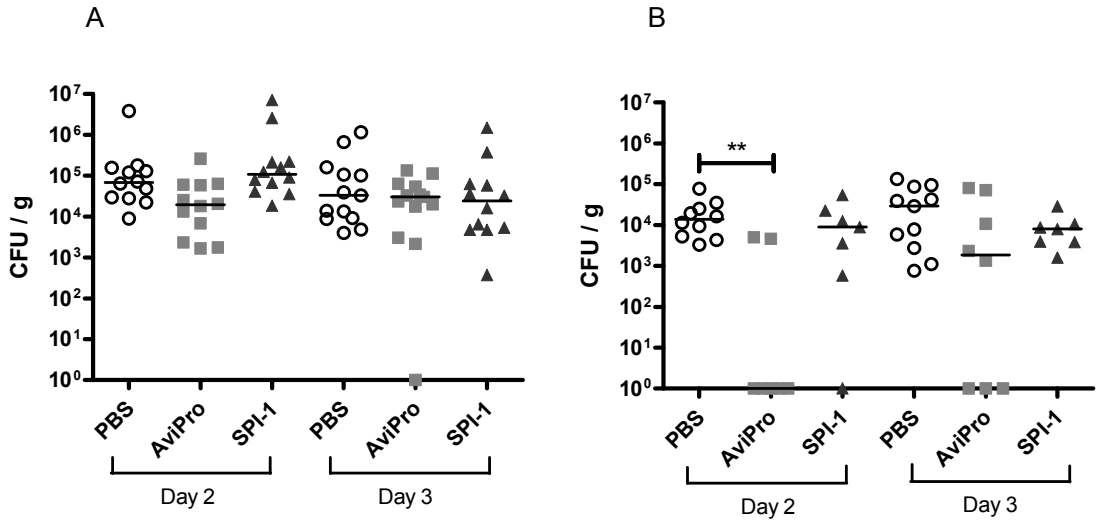

Figure 5.13: Vaccine Trial 2, Experiment II. Levels of *S*. Enteritidis in the livers of progeny obtained from vaccinated hens. Hens were vaccinated subcutaneously with either PBS (open circles), AviPro® (closed squares) or SPI-1 T3SS proteins (closed triangles) where 60 % of the seeder birds were orally inoculated with the challenge strain. Values are expressed as median colony forming units per gram (CFU / g). Bacterial levels in the livers of seeder (A) and contact exposed (B) birds on days 2 and 3 postchallenge (10^8 CFU). **, $P < 0.01$.

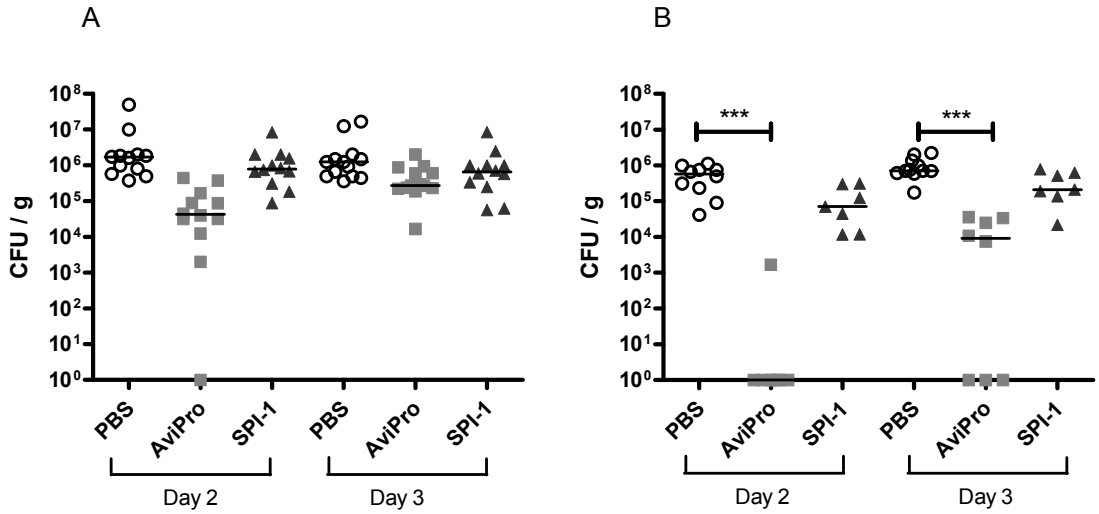

Figure 5.14: Vaccine Trial 2, Experiment II. Levels of *S*. Enteritidis in the spleens of progeny obtained from vaccinated hens. Hens were vaccinated subcutaneously with either PBS (open circles), AviPro® (closed squares) or SPI-1 T3SS proteins (closed triangles) where 60 % of the seeder birds were orally inoculated with the challenge strain. Values are expressed as median colony forming units per gram (CFU / g). Bacterial levels in the spleens of seeder (A) and contact exposed (B) birds on days 2 and 3 postchallenge (10^8 CFU). ***, $P < 0.001$.

In an effort to better simulate natural infection, in experiment III, only 10 % of the chicks (seeder birds) obtained from the hens were orally challenged while the remaining 90 % were allowed to commingle with the rest of the flock (contact exposed birds). Like experiments I and II, the levels of cecal colonization were not affected as a result of vaccination with SPI-1 proteins (Figures 5.15A and 5.15B). Interestingly, the colony counts in the liver homogenates of the progeny (contact exposed group) from the SPI-1 vaccinated hens were significantly lower ($P < 0.05$) than the progeny from the PBS vaccinated group two days after bacterial challenge (Figure 5.16B). Liver bacterial colony counts were also arithmetically lower (no statistical difference) in the chicks (contact exposed group) obtained from the AviPro® vaccinated group two days postchallenge. As well, the recovery of the challenge strain from the spleen homogenates was lower in the chicks (contact exposed group) obtained from the AviPro® vaccinated group on both days postchallenge (Figure 5.17B), but not from the chicks (seeder and contact exposed groups) obtained from the SPI-1 vaccinated hens.

5.3.3 Vaccine Trial 3

To determine if SPI-1 proteins can protect older hens against *S.* Enteritidis challenge, we orally challenged the hens that were immunized in vaccine trial 2. The levels of the challenge strain in the cecal contents were significantly lower ($P < 0.05$) in the AviPro® but not the SPI-1 vaccinated groups relative to the control (Figure 5.18). However, the recovery of *Salmonella* from the internal organ homogenates as a result of direct plating was mostly below the detection limit (Figures 5.19A and 5.19B). Data from the enrichment of the aforementioned homogenates was difficult to interpret since most of the birds were negative for *Salmonella* species (data not shown). In addition, the recovery of *Salmonella* from the homogenates of ovaries obtained from immunized hens was negative based on direct plating and enrichment (data not shown). Likewise, we were not able to detect *Salmonella* in the blood samples obtained from these hens after enrichment (data not shown).

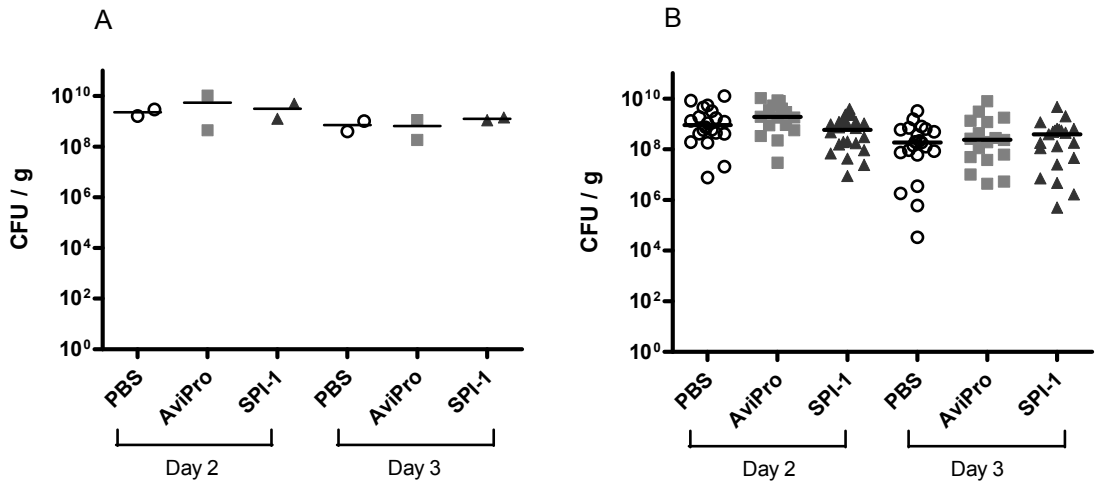

Figure 5.15: Vaccine Trial 2, Experiment III. Levels of *S*. Enteritidis in the cecal contents of progeny obtained from vaccinated hens. Hens were vaccinated subcutaneously with either PBS (open circles), AviPro® (closed squares) or SPI-1 T3SS proteins (closed triangles) where 10 % of the seeder birds were orally inoculated with the challenge strain. Values are expressed as either mean (A) or median (B) colony forming units per gram (CFU / g). Bacterial levels in the cecal contents of seeder (A) and contact exposed (B) birds on days 2 and 3 postchallenge (10^8 CFU).

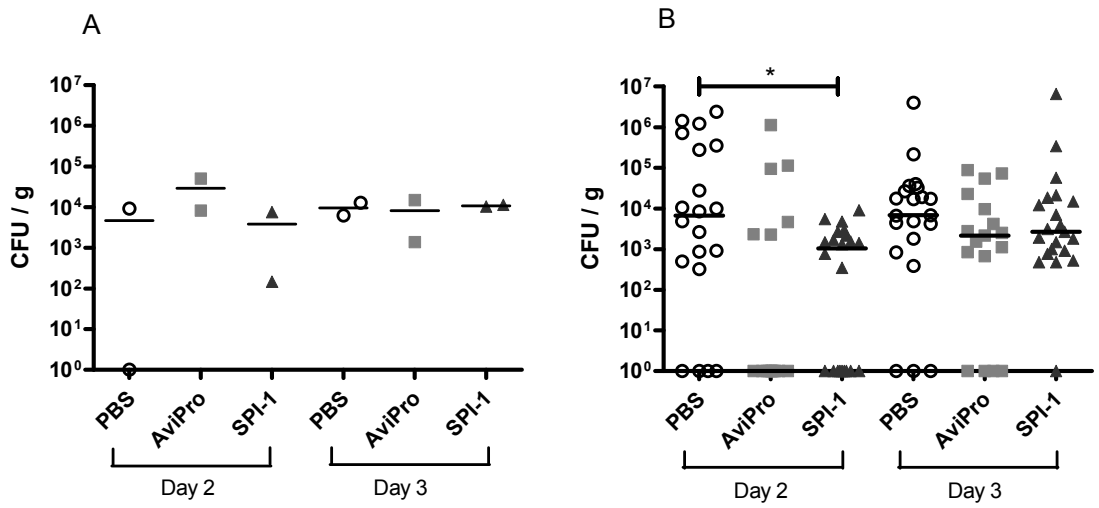

Figure 5.16: Vaccine Trial 2, Experiment III. Levels of *S*. Enteritidis in the livers of progeny obtained from vaccinated hens. Hens were vaccinated subcutaneously with either PBS (open circles), AviPro® (closed squares) or SPI-1 T3SS proteins (closed triangles) where 10 % of the seeder birds were orally inoculated with the challenge strain. Values are expressed as either mean (A) or median (B) colony forming units per gram (CFU / g). Bacterial levels in the livers of seeder (A) and contact exposed (B) birds on days 2 and 3 postchallenge (10^8 CFU). *, $P < 0.05$.

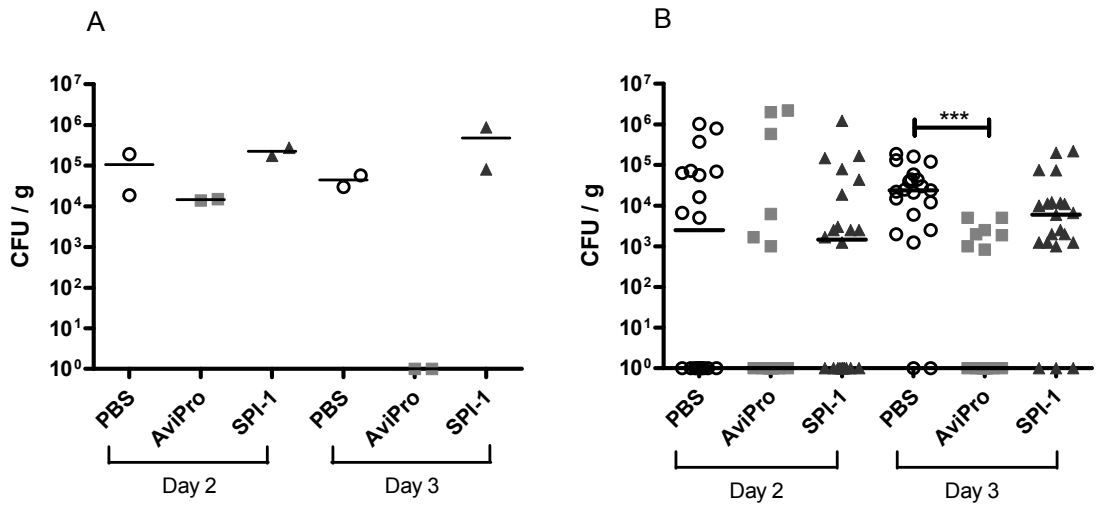

Figure 5.17: Vaccine Trial 2, Experiment III. Levels of *S*. Enteritidis in the spleens of progeny obtained from vaccinated hens. Hens were vaccinated subcutaneously with either PBS (open circles), AviPro® (closed squares) or SPI-1 T3SS proteins (closed triangles) where 10 % of the seeder birds were orally inoculated with the challenge strain. Values are expressed as either mean (A) or median (B) colony forming units per gram (CFU / g). Bacterial levels in the spleens of seeder (A) and contact exposed (B) birds on days 2 and 3 postchallenge (10^8 CFU). ***, $P < 0.001$.

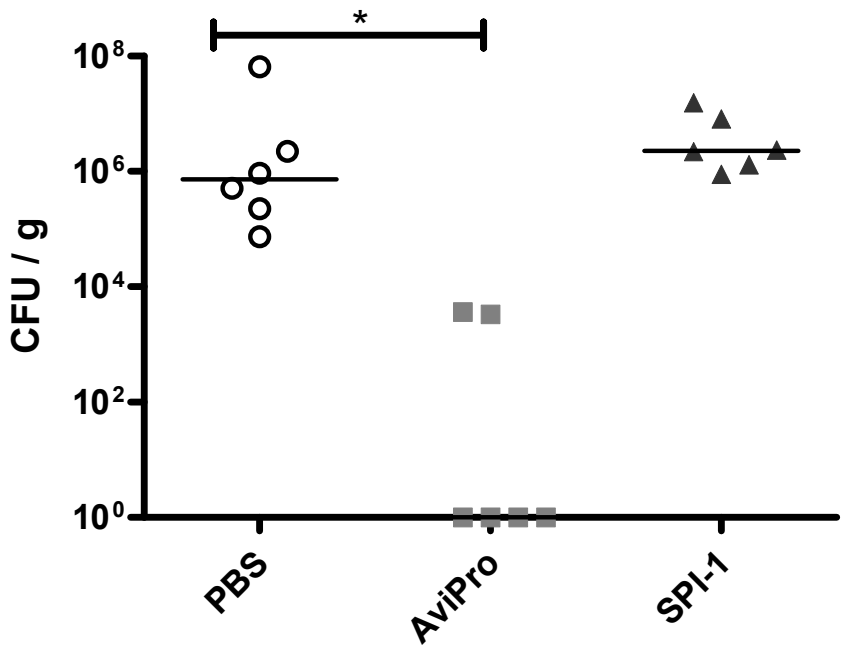

Figure 5.18: Vaccine Trial 3. Median levels of *S.* Enteritidis expressed as colony forming units per gram (CFU / g) in the cecal contents two days following oral challenge (10^8 CFU) in hens vaccinated subcutaneously with either PBS, SPI-1 T3SS proteins or AviPro®. *, $P < 0.05$.

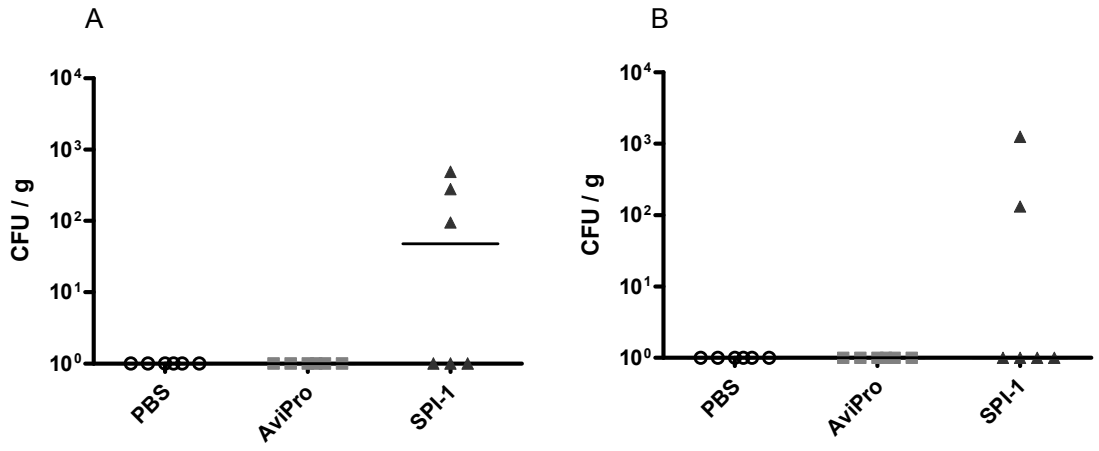

Figure 5.19: Vaccine Trial 3. Median levels of *S.* Enteritidis expressed as colony forming units per gram (CFU / g) in the livers (A) and spleens (B) two days following oral challenge (10^8 CFU) in hens vaccinated subcutaneously with either PBS, SPI-1 T3SS proteins or AviPro®. *, $P < 0.05$.

5.4 Discussion

Salmonella Pathogenicity Island 1 has been established as a major virulence determinant in *Salmonella* pathogenesis. Several studies have shown that SPI-1 is important during the process of infection in chickens [87, 89, 90, 130]. We have also recently shown that SPI-1 enhances invasion in chicken small intestinal and cecal tissue explants and its absence causes a delay in systemic infection in chickens [188]. Not only has the SPI-1 encoded secretion system been associated with virulence in chickens, it has also been implicated in pathogenesis in the bovine, murine and porcine models of infection [127, 195, 200, 201, 208]. Moreover, recent in vitro work suggests that the SPI-1 T3SS mediates intimate attachment to epithelial cells [38] and that antibodies directed against this system block *S*. Enteritidis entry into polarized Caco-2 cells (unpublished data). Taken together, this strongly indicates that the aforementioned secretion system is an important therapeutic target to control infection by this pathogen. Hence, the goal of this work was to test the potential of SPI-1 proteins as vaccine candidates against *S*. Enteritids challenge in chickens.

To our knowledge, prior to this work, only one study was performed in pigs to evaluate the efficacy of proteins secreted by the SPI-1 T3SS as vaccine targets [250]. The authors of that study used crude extracts of total SPI-1 secreted proteins to vaccinate pigs and found that the recovery of *S*. Typhimurium from intestinal contents was lower in the ileum and colon of vaccinates, but not in the intestinal lymph nodes and mucosa. However, they found that the group vaccinated with a *prgH* mutant (SPI-1 secretion defective) also had lower levels in the intestinal contents, suggesting that the protection observed was not SPI-1 specific. The fact that the vaccine based on secreted proteins from the wild type strain did not confer protection is not surprising since the SPI-1 effector proteins are injected directly into the host cell cytosol [24]. Consequently antibodies directed against these effector proteins may not be protective against *S*. Typhimurium challenge.

In the present study, we used structural proteins of the SPI-1 secretion system as vaccine candidates, since these proteins are present on the surface of the bacterial cell and hence may be more "visible" to the host immune system relative to the effector proteins, which are secreted into host cells. PrgI and SipD were used for vaccine trial 1, since they form part of the SPI-1 encoded secretion apparatus that is exposed on the surface of the bacterial cell [24]. Further, recent in vitro experiments have suggested that the tip of the SPI-1 secretion system, SipD, is a critical player in infection of epithelial cells (unpublished data) [38]. In vaccine trial 1, we demonstrated for the first time, that vaccination of chickens with PrgI and SipD significantly reduced levels of the challenge strain in the livers of the vaccinated group, while a similar trend, though not statistically significant, was observed in the spleens of the birds. The reduction in the levels of *S*. Enteritidis in the livers of chickens vaccinated with PrgI and SipD was about 1000 fold relative to the control groups. This is comparable with data from other vaccine efficacy studies where a similar reduction was observed after challenge with either *S*. Enteritidis or *S*. Typhimurium [175, 249, 251]. In addition, both PrgI and SipD induced strong IgG titers. The fact that we observed lower levels of *S*. Enteritidis in the internal organs based on direct plating and not from enrichment indicates that immunization with the SPI-1 proteins reduced the bacterial load in the vaccinates but did not affect the number of birds positive for *Salmonella*.

In order to further test the protective effect of SPI-1 antigens, we immunized hens with a combination of SPI-1 proteins and challenged their progeny (vaccine trial 2) with *S*. Enteritidis. We used a seeder model of infection because this model comes closer to natural infection compared to a high oral challenge dose and is well established [248, 249]. Three independent batches of progeny were tested where we challenged 70 % (experiment I), 60 % (experiment II) or 10 % (experiment III) of the chicks in each group (seeder birds), respectively. Our results demonstrate that vaccination with SPI-1 proteins or AviPro® (positive control) resulted in significant antibody titers in both sera from the hens and egg yolks, but did not have a major effect on the levels of the challenge strain in the progeny (seeder birds). This is not surprising since these birds were orally inoculated with 10^8 CFU of *S*. Enteritidis and this is a high dose for day old chicks. However, lower

numbers ($P < 0.05$) of *S*. Enteritidis were observed in the livers of progeny immunized with SPI-1 proteins on day two postchallenge in experiment III, but not in the other samples nor in experiments I and II. A possible hypothesis for this finding could be that SPI-1 may not play a major role in *S*. Enteritidis virulence in day old chicks. This notion is supported by the observation in a study where *S*. Typhimurium SPI-1 mutants were not impaired in the colonization of chicken ceca or in systemic infection of day old chicks [89]. Alternatively, the antibody titers to the SPI-1 antigens may not be high enough in the progeny from the vaccinated hens to induce protection as in vaccine trial 1. The fact that the levels of bacteria in the cecal contents of progeny from SPI-1 vaccinates were similar to the levels in the control group is in line with our previous observations where we demonstrated that deletion of SPI-1 does not affect cecal colonization [188]. The groups vaccinated with AviPro® in experiments II and III had significantly lower levels of the challenge strain in the internal organs, suggesting that progeny from immunized hens can be protected using a seeder model of infection. Similarly, oral challenge of the immunized hens (vaccine trial 3) resulted in lower levels of *S*. Enteritidis in the cecal contents of the AviPro® vaccinated group, but not the SPI-1 vaccinated group. However, data from the livers and spleens of all the hens, based on both direct plating and enrichment, was difficult to interpret since most of the birds were negative for *Salmonella*. This is not unusual since these birds are much older and are known to be more resistant to infection by *S*. Enteritidis. Taken together, this suggests that SPI-1 antigens play a minor role in protection against systemic disease via passive immunity based on our vaccine formulation and route of vaccine delivery.

The use of T3SS as targets for intervention strategies has also been used in other Gram negative pathogens including, *Escherichia coli*, *Yersinia* species, *Shigella* species and *Pseudomonas* species [252]. Antibodies against the major T3SS components of the aforementioned pathogens have shown to inhibit their virulence associated functions in vitro [231, 253, 254]. As well, vaccination with T3SS proteins of these pathogens has also resulted in protection from bacterial challenge in different animal models, including mice, rabbits, guinea pigs and cattle [255-258]. Additionally, this strategy of targeting T3SS has led to the development of a commercially available vaccine (Econiche™) that

protects cattle against *E. coli* O157:H7 infection [258, 259], which is the first licensed vaccine based on T3SS. Although *S*. Enteritidis employs a different strategy for causing infection compared to *E. coli* and the other pathogens mentioned above, the SPI-1 T3SS is an important virulence factor during *Salmonella* pathogenesis in chickens as well as other animal models of infection [188, 195, 197, 200]. Therefore, the *Salmonella* T3SS proteins represent good vaccine candidates for immunization of chickens.

The protection of chickens from *S*. Enteritidis systemic infection observed in this study is mainly based on IgG antibody titers to the SPI-1 T3SS proteins in the sera of vaccinated birds. Since *Salmonella* is an intracellular pathogen, it would be expected that a cell-mediated response is critical for clearance of infection. Based on studies in mice, the prevailing view is that cell-mediated immunity is a critical component of the host response to *Salmonella* infection relative to the humoral response [170, 260]. However, mice lacking B cells are able to clear infection of attenuated *Salmonella* strains but not of virulent strains. This clearly demonstrates that antibodies are important in the immune response to *Salmonella* [261, 262]. In chickens, the role of cell-mediated immunity is not as well defined, since fewer immunological tools are available. However, *Salmonella* infections have always been associated with high antibody titers in the sera (IgG) as well as in the intestinal mucosa (IgA) [156]. Similarly, infections by non-host adapted *Salmonella* strains have also been associated with up-regulation of T cells and IFNγ levels in chickens, suggesting that cellular immunity also plays a role [147, 155]. As in mice, B cell depleted chickens also possess the ability to effectively clear *Salmonella* infections [158]. Nevertheless, the role of antibodies has not been excluded [156]. Further, several killed *Salmonella* vaccines are currently in use, which mainly induce an antibody response and confer protection against oral *Salmonella* challenge [170]. Hence, the humoral response alone may not eliminate *Salmonella* species, but can significantly reduce the levels of *Salmonella* species in chickens which will ultimately improve human health.

In summary, we have shown for the first time that immunization of chickens with SPI-1 structural proteins results in high antibody titers and confers protection against *S*.

Enteritidis systemic infection. Our data also suggest that the SPI-1 based vaccine did not have a significant effect on the levels of *Salmonella* in the progeny from vaccinated hens using a seeder model of infection. Thus, PrgI and SipD may form important components of subunit vaccines that are used for reducing the prevalence of *S*. Enteritidis in poultry flocks. Further work needs to be done to investigate the protective capacity of the SPI-1 antigens possibly via mucosal delivery and in combination with different vaccine adjuvants.

6.0 GENERAL DISCUSSION AND CONCLUSIONS

6.1 General Discussion

S. Enteritidis continues to be a major source of human *Salmonella* infections worldwide [9]. This is largely the result of the consumption of contaminated poultry meat and eggs [10]. Therefore, reducing the levels of *S*. Enteritidis in chickens is a critical component of a given *Salmonella* control program [160]. Vaccination, along with other intervention strategies, has proven to be an effective tool in lowering the levels of *S*. Enteritidis in chickens in the U.K., which has consequently reduced the rates of human *S*. Enteritidis infections [10]. However, the vaccines currently available have not always been consistent in reducing the levels of *S*. Enteritidis. In addition, the vaccines consist of either live attenuated strains which have safety concerns associated with them or are killed *Salmonella* strains whose protective antigens are not well defined. As well, few vaccines provide cross protection against the different *Salmonella enterica* serovars. Hence, there is still a need for vaccines that are safe, efficacious and well defined [170].

Many studies have shown that the SPI-1 T3SS is an important virulence determinant during *S. enterica* pathogenesis [86, 127, 128, 208]. However, this has been observed mainly in the mouse and bovine models of infection with *S*. Typhimurium. In chickens, the role of SPI-1 has not been well studied. Therefore, we hypothesized that the SPI-1 T3SS is important for virulence in chickens and that proteins associated with this system could be used as vaccine candidates to protect chickens against *S*. Enteritidis oral challenge.

In order to study the role of SPI-1 in invasion, we constructed ΔSPI-1 and Δ*invG* mutants. The mutants were impaired in the secretion of effector proteins, as determined by Western blots using anti-SipD serum. To investigate the invasive properties of these mutants, we used polarized Caco-2 cells. The use of polarized cells was very useful since these cells form well defined apical and baso-lateral compartments that closely mimic the actual intestinal epithelial layer [190]. Data from the gentamicin protection assays

indicated that both ΔSPI-1 and Δ*invG* mutants were less invasive compared to the wild type strain. This was in agreement with other reports which have demonstrated that SPI-1 is important for invasion of tissue culture cells [197, 212-214]. To further study the effect of the deletion of SPI-1, we used chicken small intestinal and cecal tissue. This ex-vivo model was very valuable since it allowed us to evaluate the role of SPI-1 during infection in the chicken gastrointestinal tract. The results suggested that the deletion of SPI-1 reduced the invasiveness of the mutant strain. However, the difference in invasion between the mutant and wild type strain was smaller compared to the difference using polarized Caco-2 cells. The results from the tissue explants, along with the findings from the polarized cells imply that *S*. Enteritidis SPI-1 is important for invasion of intestinal tissue, especially in chickens.

The ΔSPI-1 strain was also tested in chickens. Oral challenge of two-week-old chickens revealed that the colonization of the cecal contents on days 1-4 post challenge was not affected by the absence of SPI-1. This was in agreement with one study [89] but not with others [88, 130]. It is possible that a difference may be observed when the colonization is monitored over longer time periods. The levels of *S*. Enteritidis were lower in the livers and spleens of the ΔSPI-1 challenged group relative to the wild type challenged group. This finding indicated that SPI-1 also played a role in spreading systemically in chickens. Our observations are in accordance with a recent study that examined the role of five major *S*. Enteritidis pathogenicity islands where the authors illustrated that the absence of SPI-1 reduced systemic infection but did not affect colonization of the ceca [263]. Taken together, the aforementioned experiments establish that the *S*. Enteritidis SPI-1 T3SS is an important virulence determinant in vitro, in ex vivo tissue samples and in chickens, thus confirming our hypothesis.

The prevailing view has been that SPI-1 is essential for intestinal pathogenesis in different animal models. In the mouse model of infection, it has been observed that SPI-1 mutants were recovered at lower levels from intestinal contents and systemic sites relative to the wild type strain [198]. Likewise, other groups have reported that the presence of a functional SPI-1 T3SS is important for the induction of intestinal

inflammation and histopathological changes in the streptomycin-treated mouse model of infectious enterocolitis [128, 197, 199]. Recent work indicates that SPI-1 is critical in causing enterocolitis in mice at early timepoints but plays only a minor role in intestinal disease at later stages of infection [136]. In addition, several reports have demonstrated that *S*. Typhimurium SPI-1 mutants can cause disease in a SPI-1 independent manner in the murine model of infection [127, 201]. As well, it has been shown that SPI-1 mutants retain their ability to invade M cells in a murine gut loop model [224]. The aforementioned studies illustrate that *Salmonella* is able to breach the intestinal epithelial layer by employing other mechanisms besides the SPI-1 T3SS. Therefore, *Salmonella* translocates across the intestinal epithelial layer mainly by three different routes (Figure 6.1): (i) invasion of enterocytes (SPI-1 dependent), (i) uptake through M cells, (iii) uptake through dendritic cells [136]. The fact that we did not see an effect of the absence of SPI-1 on cecal colonization in chickens suggests that *S*. Enteritidis mainly uses SPI-1 independent mechanisms for translocating across the chicken gastrointestinal tract. Further, our data imply that SPI-1 also plays a role in systemic infection in chickens. This is not surprising since two recent studies have shown that SPI-1 effector proteins were expressed during systemic infection in mice [86, 264]. Although, the murine model of salmonellosis is different from chickens, these findings suggest that the role of SPI-1 can also be extended to the systemic phase of infection.

Prior to testing the efficacy of the SPI-1 T3SS proteins in protection, we wanted to determine if sera against these proteins could block invasion of Caco-2 cells by *S*. Enteritidis. Interestingly, serum against a fraction containing SPI-1 T3SS secreted proteins significantly inhibited invasion of *S*. Enteritidis relative to the control group. This was a vital finding since it suggested that antibodies directed against SPI-1 T3SS related components could be used as a therapeutic strategy for controlling the spread of this pathogen. In order to identify which components were involved in the invasion inhibition effect, we tested the effect of sera against different structural and secreted T3SS proteins. Our results indicate that anti-SipD serum blocks *S*. Enteritidis entry *in vitro*, while sera against InvG, PrgI, SipA, SipC, SopB, SopE and SopE2 did not. To verify that the invasion inhibition effect was SipD specific, we incubated anti-SipD

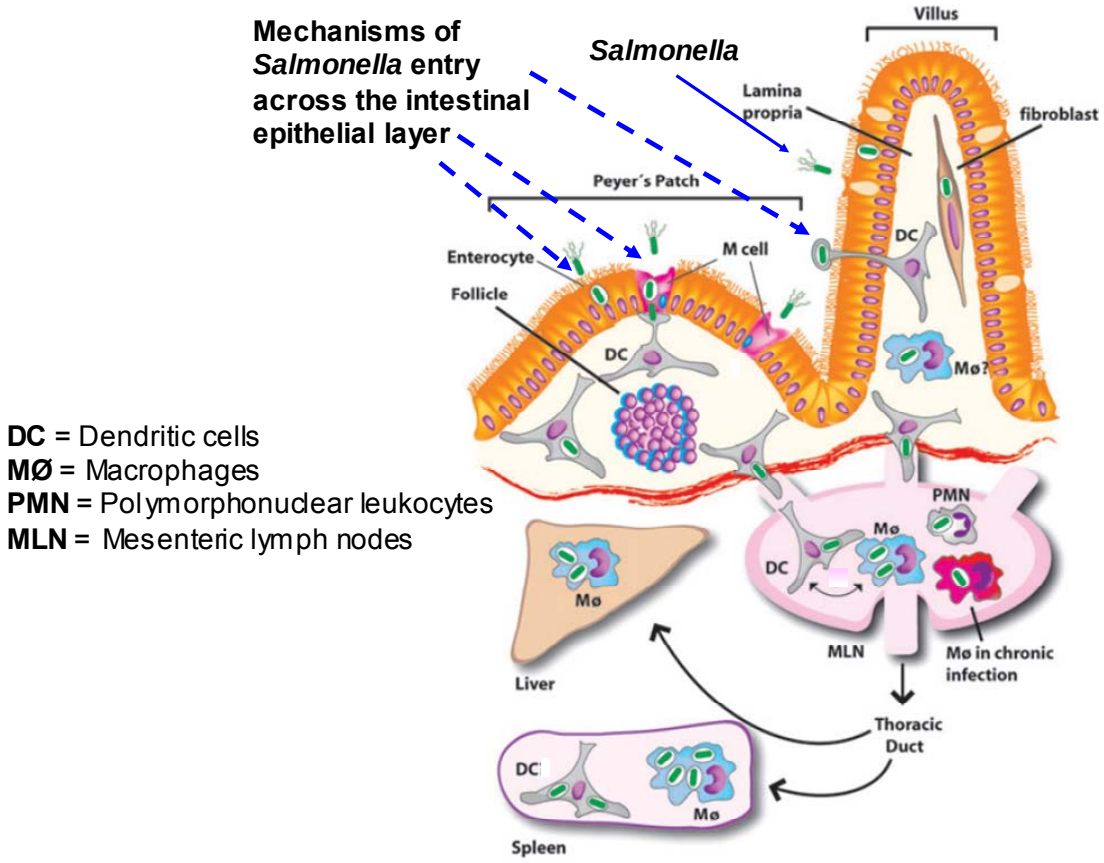

Figure 6.1. Schematic representation of *Salmonella* entry across the epithelial layer based on studies in mice. *Salmonella* translocates across the intestinal epithelial surface through enterocytes, M cells and dendritic cells. *Salmonella* then travels through the lymphatic system to the liver and spleen via macrophages and dendritic cells. Diagram was adapted and modified from [265].

serum with a strain lacking *sipD*. As expected, the sera had no effect on the invasiveness of the mutant strain. Further, we depleted SipD specific antibodies from anti-SipD serum and found that the depleted serum restored *S*. Enteritidis invasion. The fact that anti-SipD serum blocked *S*. Enteritidis entry is also in agreement with a recent report which suggests that SipD forms the tip of the SPI-1 T3SS apparatus and that it is involved in intimate attachment to epithelial cells [38].

In order to evaluate the potential of SPI-1 T3SS proteins as vaccine candidates, we vaccinated chickens subcutaneously with PrgI (needle protein) and SipD. Although the anti-PrgI serum did not affect the invasion of *S*. Enteritidis *in vitro*, it is possible that antibodies against this protein may be important in protection since the PrgI needle protrudes about 50 nm above the surface of the bacterial cell [17]. Vaccination with the aforementioned proteins induced a significant antigen specific serum IgG response and resulted in lower levels of the challenge strain in the livers (3 log units lower on day 4 postchallenge) and spleens of vaccinated birds. This suggested that these proteins were immunogenic and were capable of conferring protection against systemic spread of *S*. Enteritidis. The PrgI and SipD proteins provided better protection compared to a study in which *S*. Enteritidis type I fimbriae were used as a subunit vaccine where there was no difference in the levels of the challenge strain between the internal organs of vaccinated and control groups [184]. We were not able to compare the level of protection we observed from systemic infection with other vaccination studies in chickens that have used subunit vaccines like outer membrane proteins and the FliC flagella protein since these studies did not examine the levels of *S*. Enteritids in the internal organs [182, 183, 185]. However, in our study vaccination with PrgI and SipD did not affect the levels of *S*. Enteritidis in the cecal contents. This is in line with a study in which vaccination of chickens with *S*. Enteritidis type I fimbriae did not affect the levels of the challenge strain in the cecal contents [184]. On the contrary, this is not in agreement with the aforementioned vaccination study where chickens were vaccinated with the FliC protein resulting in a 3 log unit decrease in the colonization of the cecal contents by *S*. Enteritidis [185]. Likewise, our data is not in agreement with a report which demonstrates that immunization of chickens with *S*. Enteritidis outer membrane proteins leads to a 2-3 log

unit decrease in the colonization of the ceca by *S*. Enteritidis [183]. This could be explained by the fact that the SPI-1 T3SS may not be very important for breaching the intestinal epithelial surface in chickens and that *S*. Enteritidis might be using SPI-1 indpendent mechanisms to invade the chicken gastrointestinal tract.

Similarly, we also tested the efficacy of SPI-1 T3SS proteins in conferring protection in progeny obtained from vaccinated hens using a seeder model of infection. This would allow us to determine if these antigens could be used for immunization of breeders. Moreover, a seeder model comes very close to the natural situation that the chickens would encounter at a poultry facility. Vaccination with the T3SS proteins induced significant antibody titers in the hen sera as well as in the egg yolks. However, there was significant protection in the livers of progeny obtained from vaccinated hens only in experiment III on day 2 postchallenge in the contact exposed group. This could be explained by the fact that the antibody titers to the SPI-1 proteins in the progeny from vaccinates may not be sufficient to induce protection. Alternatively, SPI-1 may not play an important role in day old chicks. In order to induce better protection in progeny obtained from vaccinated hens, oral immunization of the hens with the aforementioned antigens (possibly with a combination of subcutaneous immunization) may be more efficacious since this would induce a mucosal immune response as well. Mucosal IgA antibodies specific for SPI-1 T3SS proteins may not necessarily prevent SPI-1 mediated translocation since *S*. Enteritidis can breach the gastrointestinal tract of chickens via non-SPI-1 mediated mechanisms. Further, data from our invasion inhibition study demonstrated that anti-SipD serum inhibited invasion of polarized Caco-2 cells in vitro. Although we were able to inhibit *S*. Enteritidis entry in vitro, we were not able to completely block entry of the bacterium. Therefore, a SPI-1 T3SS protein specific IgA antibody response alone may not be sufficient for providing protection against *S*. Enteritidis in chickens.

To take these results further, it will be important to vaccinate chickens with PrgI and SipD alone and in combination to determine if both proteins are involved in protection against systemic infection. Once this has been determined, the vaccine

candidate(s) should be delivered orally, possibly in combination with a subcutaneous immunization, to determine if a mucosal immune response can enhance the protection against *S*. Enteritidis. Additionally, the vaccine candidate(s) can be formulated with known adjuvants that enhance not only the antibody response, but also the cell-mediated immune response since *S*. Enteritidis is an intracellular pathogen. An optimized vaccine formulation may then be used for testing for protection against other non-host adapted *S. enterica* serovars like *S*. Typhimurium since it is also highly prevalent in chickens.

In summary, the results suggest that the SPI-1 T3SS is an important virulence factor in chickens and that the T3SS proteins can form important components of a subunit vaccine to reduce the prevalence of *S*. Enteritidis in poultry, confirming our initial hypothesis.

6.2 General Conclusions:

- *S*. Enteritidis ΔSPI-1 and Δ*invG* mutants are impaired in the secretion of effector proteins and in invasion.
- *S*. Enteritidis ΔSPI-1 is less invasive compared to the wild type strain in chicken small intestinal and cecal tissue.
- The absence of SPI-1 causes a delay in systemic infection in chickens but does not affect the colonization of the cecal contents.
- Anti-SipD serum blocks *S*. Enteritidis entry in polarized Caco-2 cells.
- Immunization of chickens with PrgI and SipD induces antigen specific IgG antibody responses and confers protection from systemic infection.
- Vaccination of hens with SPI-1 T3SS proteins induces antigen specific IgG antibody responses but does not confer significant protection from systemic infection in progeny derived from the hens.

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